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Impact of obesity on hypothalamic microRNAS: from pathophysiology to gene therapy approach

Helena Maria da Silva Leal

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Impact of obesity on hypothalamic microRNAs: from pathophysiology to gene therapy approach

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob orientação da Doutora Lígia Sousa-Ferreira (Centro de Neurociências e Biologia Celular) e da Professora Doutora Emília Duarte (Departamento de Ciências da Vida, Universidade de Coimbra)

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«The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" but "That's funny..."»
Isaac Asimov

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ABBREVIATIONS

3V	Third Ventricle
Ago2	Argonaute 2
AgRP	Agouti-Related Protein
ARC	Arcuate Nucleus
Ascl1/Mash1	Achaete-scute homolog 1
BBB	Blood-Brain-Barrier
BCA	Bicinchonic Acid
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CART	Cocaine and Amphetamine-Regulated Transcript
CNTF	Ciliary Neurotrophic Factor
CRH	Corticotropin-releasing Hormone
Ddx6	DEAD (Asp-Glu-Ala-Asp) box helicase 6
DGCR8	DiGeorge syndrome Chromossomal Region 8
DMN	Dorsomedial Nucleus
DTT	Dithithreitol
ECF	Enhanced Chemifluorescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
Fmr1	Fragile X mental retardation 1
GFAP	Glial fibrillary acidic protein
GFP	Green Fluorescent Protein
GTT	Glucose Tolerance Test
HEK 293	Human Embryonic Kidney 293 cells
HFD	High Fat Diet
HMGA2	HMGA2 high mobility group AT-hook 2
HPRT	Hypoxanthine Phosphoribosyl Transferase
i.c.v	intracereboventricular
Iba1	Ionized calcium-binding adapter molecule 1
IGF-1	Insulin-like Growth Factor 1
IKBKB/IKKβ	Inhibitor of Nuclear factor kappa-B kinase subunit beta

IL1β	Interleukin-1 beta
IL6	Interleukin 6
InsR	Insulin Receptor
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
LepR	Leptin Receptor
LHA	Lateral Hypothalamic Area
Lin28	Protein lin-28 homolog A
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MCH	Melanin-concentrating hormone
MEm	Median Eminence
miRISC	miRNA-induced silencing complex
miRNAs, miRs	microRNAs
mRNA	messenger RNA
MS1	Musashi1
NaCl	Sodium Chloride
ND1, Neuro D1	Neurogenic differentiation 1
NF-κB	Nuclear Factor kappa B
NPC	Neural Progenitor Cell
NPY	Neuropeptide Y
NSC	Neural Stem Cell
NTS	Solitary Tract Nucleus
OCT4	Octamer-binding Transcription factor 4
ORX	Orexin
OXY	Oxytocin
PabpC1	Poly(A) Binding Protein, Cytoplasmic 1
PBS	Phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PFA	Paraformaldehyde
PGK	Phosphoglycerate Kinase 1
PMSF	Phenylmethylsulphonylfluoride
POMC	Pro-Opiomelanocortin

pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PVDF	Polyvinylidene difluoride
PVN	Paraventricular Nucleus
qRT-PCR	quantitive Real Time-Polimerase Chain Reaction
RIPA	RadioImmunoPrecipitation Assay
SDS	Sodium Dodecyl Sulfate
SGZ	SubGranular Zone
SOCS3	Suppressor Of Cytokine Signaling 3
SOX2	(Sex determining region Y)- box 2
STAT-3	Signal Transducer and Activator of Transcription-3
SVZ	SubVentricular Zone
TBS-T	Tris-Buffered Saline Tween-20
TLR-4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor alpha
TRBP	Trans-activation Response RNA-Binding Protein
TRH	Thyrotropin-Releasing Hormone
VMN	Ventromedial Nucleus
WAT	White Adipose Tissue

Abstract

Obesity is an emerging concern in our society and it is related to high-fat diet (HFD) consumption that causes metabolic changes and alterations of body homeostasis.

In the central nervous system, the hypothalamus is the key regulator of metabolism because it controls feeding behavior, body temperature, circadian cycle, among others. Neurons in the hypothalamus receive and integrate signals from peripheral organs that will regulate physiological and behavioral functions in order to maintain body homeostasis. Importantly, hypothalamic function is altered in obesity. In fact, inflammation was observed in the first days of HFD in obesity animal models that, if continued, led to neuronal cell death in the hypothalamus.

Moreover, in the hypothalamus is present a neurogenic niche whose activity is altered depending on the energetic state. The activity of neural progenitor cells was observed to be stimulated in the first days of HFD. However a sustained high fat intake led to impaired hypothalamic neurogenesis.

MiRNAs are a class of endogenous, small, non-coding RNAs with post-transcriptional functions. MiRNAs interact with target mRNA to decrease the expression levels of corresponding protein. Alterations in miRNAs expression in peripheral organs were observed in metabolic diseases but their involvement in the central regulation of metabolism in the hypothalamus is unknown.

A previous study from our laboratory revealed altered expression levels of let-7 microRNAs in the rat hypothalamus after HFD regime. Let-7 miRNAs are a family of miRNAs involved in several cellular mechanisms including maturation during neurodevelopment, inflammatory processes and glucose metabolism. Since these pathways are impaired in the hypothalamus in obesity we hypothesize that let-7 microRNA could be the regulator of these alterations.

In this project we investigated the impact of obesity on hypothalamic microRNA pathway and explored the putative protective effect of let-7b in obesity associated metabolic alterations and neuropathological dysfunction.

We observed that let-7 microRNAs levels and related protein Lin28b are altered in the rat hypothalamus after different periods of HFD; and that these alterations are related to alterations in neurogenesis markers.

Moreover, we showed that inflammation can alter the let-7/Lin28b system in the hypothalamus, by inducing inflammation with LPS in hypothalamic slice cultures.

Furthermore, the overexpression of let-7b in the hypothalamus of mice in a HFD regime showed ameliorations regarding metabolic function, including decreased body weight gain, decreased caloric intake and improved glucose tolerance. Moreover, neuropathological impairment induced by HFD consumption in the hypothalamus was partially prevented by let-7b, in particular neuroinflammation and neurogenesis changes.

In conclusion, we identified let-7b as a new anti-obesity target warranting further studies to support its therapeutic potential.

Keywords: hypothalamus, obesity, microRNAs

Resumo

A obesidade é uma preocupação emergente da nossa sociedade relacionada com o consumo de uma dieta hipercalórica que causa alterações metabólicas e homeostáticas. No sistema nervoso central, o regulador chave do metabolismo é o hipotálamo, que controla o comportamento alimentar, temperatura corporal, ciclo circadiano, entre outros. Os neurónios do hipotálamo recebem e integram sinais de órgãos periféricos que regulam funções fisiológicas e comportamentais de modo a manter a homeostasia corporal. De destaque, em obesidade há disfunção hipotalâmica. Na verdade, a inflamação observada nos primeiros dias de dieta hipercalórica em modelos de obesidade quando continuada pode levar a morte de células neuronais no hipotálamo. Aliás, no hipotálamo está presente um nicho neurogénico cuja actividade é alterada dependendo do estado energético. Foi observada que a actividade de células neuroprogenitoras é estimulada nos primeiros dias de dieta hipercalórica. Contudo se a dieta for mantida a neurogénese fica comprometida.

MiRNAs são uma classe de RNAs endógenos, pequenos e não-codificantes com funções pós transcripcionais. MiRNAs interagem com mRNAs alvo para diminuir os níveis de expressão da proteína correspondente. Em órgãos periféricos foram observadas alterações de expressão de miRNAs em doenças metabólicas. Contudo o seu envolvimento na regulação metabólica pelo hipotálamo é desconhecido.

Um estudo anterior no nosso laboratório revelou expressão alterada de microRNAs let-7 no hipotálamo de ratos após dieta hiper calórica. A família de microRNAs let-7 está envolvida em vários mecanismos celulares incluindo maturação durante o desenvolvimento neuronal, processos inflamatórios e metabolismo da glicose. Visto que estas vias estão comprometidas no hipotálamo em obesidade a nossa hipótese é que o microRNA let-7 possa ser regulador destas alterações. Neste projecto investigamos o

efeito da obesidade na via dos microRNAs hipotalâmicos e exploramos os possíveis efeitos do let-7b em obesidade associado a alterações metabólicas e disfunção neuropatológica. Observamos que os níveis de microRNAs let-7 e proteína relacionada Lin28b estão alterados no hipotálamo de ratos que antes estiveram em dieta hipercalórica por períodos diferentes; e que estas alterações estão relacionadas com as mudanças em marcadores de neurogénese. Além disso, mostramos que inflamação consegue alterar o sistema let-7/Lin28b no hipotálamo ao induzir inflamação com LPS em culturas hipotalâmicas.

Ainda, a sobre expressão de let-7b no hipotálamo de ratinhos em dieta hipercalórica revelou melhorias relativamente à função metabólica, incluindo redução no ganho de peso, redução no consumo calórico e na melhoria na tolerância a glucose. Também, induzidos pelo consumo de dieta hipercalórica no hipotálamo danos neuropatológicos, foram parcialmente prevenidos pelo let-7b, em particular alterações na neuroinflamação e neurogénese.

Em conclusão identificamos o let-7b como um novo alvo anti-obesidade requerendo mais estudos para apoiar seu potencial terapêutico.

Palavras – Chave: hipotálamo, obesidade, microRNAs

Chapter I. Introduction

1.1 Obesity

Obesity is a metabolic disease that can escalate into severe pathologies such as diabetes and cardiac diseases [1-3], having a big impact in our society [1, 4].

Obesity is characterized by deregulation of the body homeostatic state. When the body is exposed to high caloric conditions there is an increase of energy storage in adipocytes, in a lipidic form [5]. This may cause expansion of the existent adipocytes or an increase of adipocyte number [5]. These mechanisms trigger inflammation and, in the first stages, there is a release of anti-inflammatory proteins by adipocytes. However in a continued high caloric intake, inflammation is settled and the continued increase of adipocyte tissue leads to tissue hypoxia, adipocyte death and activation of pro-inflammatory signals [5, 6]. Eventually this results in overweightness and may lead to insulin resistance by target organs [3, 5].

This inflammatory process occurs in various peripheral organs, such as skeletal muscle, liver, adipose tissue and also in the brain, more specifically in the hypothalamus [7-9]. Moreover, all these processes are regulated by hormones which can control different circuitries and thus cause obesity treatment to be much more complicated [4, 7].

1.2 Hypothalamus

The hypothalamus is a region of the brain organized in various nuclei [10]: Arcuate Nucleus (ARC), Paraventricular Nucleus (PVN), Lateral Hypothalamic Area (LHA), Ventromedial Nucleus (VMN), and Dorsomedial Nucleus (DMN) (Figure 1.1) [10].

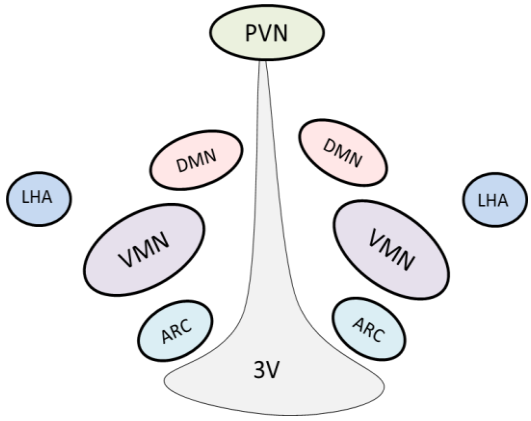

Figure 1.1 - Organization of the hypothalamic nuclei. ARC - Arcuate Nucleus, PVN - Paraventricular Nucleus, LHA - Lateral Hypothalamic Area, VMN - Ventromedial Nucleus, and DMN - Dorsomedial Nucleus.

The hypothalamus is responsible for regulation of physiological and behavioral functions, like body temperature, thirst, feeding and circadian cycle among others [11]. In fact, the control of food intake and body homeostasis relies on the hypothalamus, with the ARC being a key integrator of peripheral signals [12, 13]. This nucleus surrounds the third ventricle and is adjacent to the median eminence (MEm), where the Blood-Brain-Barrier (BBB) has distinct characteristics. In this area, the BBB allows molecule diffusion more easily because the capillaries are fenestrated and the tanycytes present do not have efficient tight junctions [14]. This characteristic allows the ARC neurons to have privileged contact with peripheral metabolic signals, circulating hormones and nutrients [10].

The ARC has two neuronal populations important for the regulation of food intake: the POMC (Pro-opiomelanocortin)/CART (cocaine and amphetamine-regulated

transcript) neurons, that are anorexigenic (decrease food intake); and, the NPY (Neuropeptide Y)/AgRP (Agouti-related protein) neurons, which are orexigenic (increase food intake) [10]. NPY positive neurons are present in various hypothalamic nuclei and other regions of the brain [15, 16], and co-localize with AgRP in the ARC (AgRP expression is restricted to the ARC) [15, 17]. POMC/CART neurons are also exclusively localized in the ARC [15]. These neurons release peptides that result of POMC processing, including melanocortins, like α-melanocyte-stimulating hormone (α-MSH), to which AgRP is an antagonist [15].

ARC neurons are considered first order neurons, since they respond directly to peripheral signals and project to second order neurons located in other hypothalamic nuclei, including PVN, DMN, VMN and LHA, [18], that in turn project to other areas of the brain [12, 18] (Figure 1.2).

The PVN integrates neuropeptide signals from different brain regions, including the ARC. Thyrotropin – releasing hormone (TRH) neurons, Corticotropin – releasing hormone (CRH) and oxytocin (OXY) neurons are located in the PVN and their activity is modulated by ARC neurons [18]. In detail, activation of POMC/CART neurons inhibits PVN activity while NPY/AgRP activation increases PVN activity, and thusly increasing food intake [18]. As for DMN apart from receiving projections from the ARC, it has NPY expressing neurons in close proximity to POMC/CART fibers [18]. NPY/AgRP and POMC/CART neurons project to the LHA modulating orexin and MCH (melanin-concentrating hormone) expressing neurons activity [18]. Orexin neurons have NPY and leptin receptors and modulate the seeking behavior according to energy availability [18]. In the VMN there are projections of NPY/AgRP neurons as well as POMC/CART neurons from the ARC [18]. Anorexigenic factor BDNF (Brain derived neurotrophic factor) is highly expressed in the VMN (and in lower rates in the

PVN) [19] having a contribution to food intake signaling. In turn, VMN neurons project to other nuclei and brain regions, such as the brainstem [10, 18].

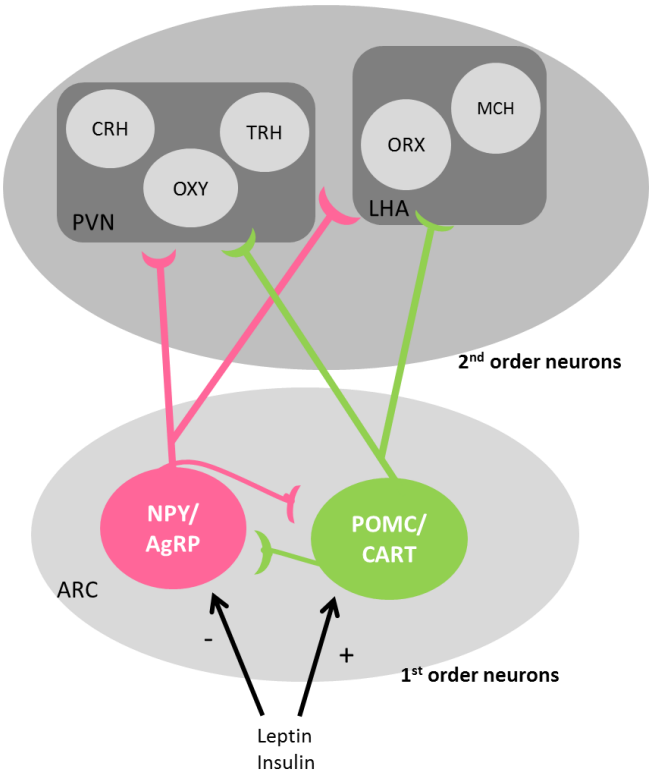

Figure 1.2 – Schematic representation of NPY/AgRP and POMC/CART neurons in the arcuate nucleus (ARC) of hypothalamus and their interaction with other hypothalamic nuclei. NPY/AgRP neurons (orexigenic neurons) and POMC/CART neurons (anorexigenic) are inhibited and stimulated, respectively by both insulin and leptin. First order neurons in the ARC, NPY/AgRP neurons and POMC/CART neurons project to second-order neurons in the PVN and LHA, which express feeding-related peptides and make the connection to the neuroendocrine system, brainstem and higher centres of the brain. (CRH - Corticotropin – releasing hormone; TRH - Thyrotropin – releasing hormone; MCH – Melanin-concentrating hormone; ORX – Orexins; OXY - Oxytocin).

The communication between hypothalamus and other regions of the brain is of extreme importance. For example, the hypothalamus communicates with the brainstem through projections from the PVN to the solitary tract nucleus (NTS). The NTS receives satiety signals from the gastrointestinal tract making it an important connection between the gut and the brain [10, 17, 18].

In conclusion, neuronal populations in the hypothalamus assemble information from peripheral organs and communicate to other brain regions to regulate homeostasis and assure physiological conditions.

1.2.1 Feeding regulation by hypothalamic circuits

Hormones released by peripheral organs modulate the activity of hypothalamic neurons to maintain body homeostasis. One of these hormones is leptin which is secreted by the adipose tissue in proportion to fat storages [10, 18, 20]. In the hypothalamus leptin receptors are present in the ARC, VMN, PVN and DMN nuclei, and specifically in POMC neurons and NPY/AgRP neurons [10, 15]. Leptin activates POMC anorexigenic neurons while reducing the activity of NPY/AgRP orexigenic neurons, resulting in a reduction of appetite [10, 15].

Another circulating hormone that modulates hypothalamic activity is insulin which is released by pancreatic β-cells in high energy level state. In the hypothalamus, insulin receptors are expressed in the ARC, DMN and PVN, and, in particular in POMC/CART neurons and NPY/AgRP neurons [10, 15]. Insulin modulates ARC neurons, activating POMC anorexigenic neurons and inhibiting NPY/AgRP orexigenic neurons [10, 15].

In the gastrointestinal system it is released an orexigenic factor, ghrelin, which activates NPY/AgRP neurons [10]. Ghrelin is a physiological meal regulator because high levels of ghrelin trigger the initiation of the meal [21]. Moreover, nutrients such as glucose can regulate feeding behavior, glucose levels rise after feeding and signal a satiety condition; such happens in the ARC and VMH where glucose-sensing machinery is present [10].

In a general aspect integrating these hormones, in high energetic state leptin and insulin are released and will activate POMC neurons, while inhibiting NPY/AgRP

neurons, resulting in a compensatory reduction of food intake [15, 16, 18]. On the other hand, in low energy levels, ghrelin is released and activates orexigenic NPY neurons, and along with NPY stimulates the production of AgRP, an antagonist of melanocortin receptors, inhibiting anorexigenic POMC neurons, stimulating food intake [15].

In the obese state is observed insulin and leptin resistance. Inflammation activated by the fatty acids in the diet compromises both insulin and leptin signaling pathway [22]. Regarding insulin, it is observed insulin signaling pathway impairment thus preventing a correct response leading to insulin resistance [23, 24]. Leptin levels are elevated in obesity, and accompany the development of the disease. Leptin resistance may be caused by impairment to reach the receptors in the hypothalamus and impaired signaling pathway [25]. Furthermore, evidence state leptin resistance to be specific of the ARC [26].

1.2.2 Hypothalamic alterations in obesity

In obesity animal studies it has been observed a deregulation of the hypothalamic feeding circuits correlated with inflammation [8]. This was corroborated in humans with MRI studies revealing hypothalamic inflammation in obese subjects [9].

In rodents exposed to HFD (high-fat diet), after the first days of diet, there is activation of pro-inflammatory factors, such as cytokines, TNF-α (tumor necrosis factor-α), SOCS3 (suppressor of cytokine signaling 3), and interleukins (IL1β and IL6) in the hypothalamus [8]. The diet components, specifically the saturated fatty acids, are able to activate TLR4 (Toll-like receptor-4), which leads to activation of TNF-α, and interleukins [27]. TLR4 is an activator of IKKβ/NF-kβ, a pathway involved in insulin and leptin resistance through SOCS3 activity [22-24, 28]. SOCS3 is activated upon leptin receptor activation in order to limit the activity of leptin signal; however, in

obesity, SOCS3 is highly expressed being associated to leptin resistance [25, 26, 28]. Interestingly, after the increased hypothalamic expression levels of SOCS3, IL6, TNF-α, IKBKB (Inhibitor of Nuclear factor kappa-B kinase subunit beta) in the first days of an HFD, it was observed a reduction during the following 4 weeks [9]. Nevertheless, an increase of mRNA expression levels of inflammatory mediators was observed thereafter [9]. Further studies, show that the suppression of IKKβ/NF-κB pathways in the medial-basal hypothalamus offer resistance to obesity under high fat diet conditions [28].

Additionally, HFD triggers gliosis, the process of activation and proliferation of microglia and astrocytes [9]. This activation occurred within the first week of HFD as observed by GFAP (Glial Fibrillary Acidic Protein), astrocytes marker and Iba1 (ionized calcium-binding adapter molecule 1) microglia marker, staining in rodent hypothalamus [9]. Astrocytes activation had the same pattern as cytokines expression in the hypothalamus, with a decrease after the first week and a return to high values after a month of HFD [9]. Microglia is activated in the first days of HFD and is then maintained. Besides, it was observed a correlation between microglia accumulation and fat gained [9].

Furthermore, with a continuous exposure to HFD (1 month) the expression levels of these mediators increase along with more severe metabolic conditions [9]. Interestingly, inflammation in obesity occurs firstly in the hypothalamus than in peripheral organs [9].

Furthermore, in rodent HFD models it was observed an increase of autophagosomes and up-regulation of chaperones as a response to endoplasmic reticulum stress in the hypothalamus [9]. This observation suggest a defense strategy responding to the HFD [9]. However it appears that the protective effect in the early state is not enough to overcome the effect of a continuous exposure [9]. However, in long term HFD was observed endoplasmic reticulum stress and reduction of autophagy activity that are

related with the activation of IKKβ/NF-κB pathways that also leading to energy imbalance and both insulin and leptin resistance [28, 29]. Additionally, overactivation of IKKβ/NF-κB in mice in chow regime led to glucose tolerance and hyperinsulinemia, overeating, weight gain, and associated neuronal loss, namely loss of POMC neurons [30]. Also, in long term exposure to HFD there is a decrease of POMC and NPY neurons population, almost exclusive in the ARC [8]. More particularly, POMC neurons exhibited high apoptotic rates [9]. These neurons, due to their anorexigenic effect, are very important for protection against obesity [9].

Hence, obesity models present deregulation of the body physiology since the hypothalamus will not be able to respond according to energy availability [31]. This may be the cause or the consequence of an increase inflammatory response to a hyper caloric diet [9].

1.3 Adult hypothalamic neurogenesis

1.3.1 Neurogenesis

Neurogenesis, the process of generating new neurons, occurs during development and also in the adult brain [11].

In the adult brain are present neural stem cells (NSC's) with the capacity to generate neural and glia lineages. These cells have self-renewal ability, maintaining a pool of stem cells, which is regulated by several transcription factors. NSC's proliferate originating neuroprogenitor cells (NPC's), with proliferative ability and maintaining an undifferentiating state by signalling of transcription factors, like SOX2 and proliferative protein, PCNA. Pro-differentiating factors, prevent cell proliferation leading to early differentiation state, resulting in newly formed neurons [32, 33].

The most studied locations for neurogenesis are the SVZ (subventricular zone of the lateral ventricles) and the SGZ (subgranular zone of the dentate gyrus of the hippocampus), but other zones have that ability, including the basal forebrain, striatum, amygdala, substantia nigra and hypothalamus [11].

1.3.2 Hypothalamic Neurogenic Niche

In the hypothalamus the ependymal cell layer surrounding the third ventricle (3V) has been pointed as a neurogenic niche [34] (Figure 1.3). Also, this is a highly vascularized zone, a characteristic shared between neurogenic niches [11]. The hypothalamic ventricular zone in the adult is constituted of: glia-like ependymal cells, named tanycytes, ependymal cells and subependymal cells [11, 35]. The tanycytes project to hypothalamic nuclei, including the ARC [36, 37] and have been identified to have the ability to proliferate and originate new neurons [38]. The subenpendymal cells of the hypothalamus also have proliferative capacity like the ones in SVZ [39]. These two cells populations are considered the neuroprogenitor cells (NPC's) of the hypothalamus [34].

There is not a consensus about the hypothalamic neurogenic niche location because two zones have been identified: (1) the lateral hypothalamic ventricular zone with proliferative tanycytes and proliferative subependymal cells and, (2) the ventral hypothalamic ventricle zone (Median Eminence - MEm) with proliferative tanycytes [37].

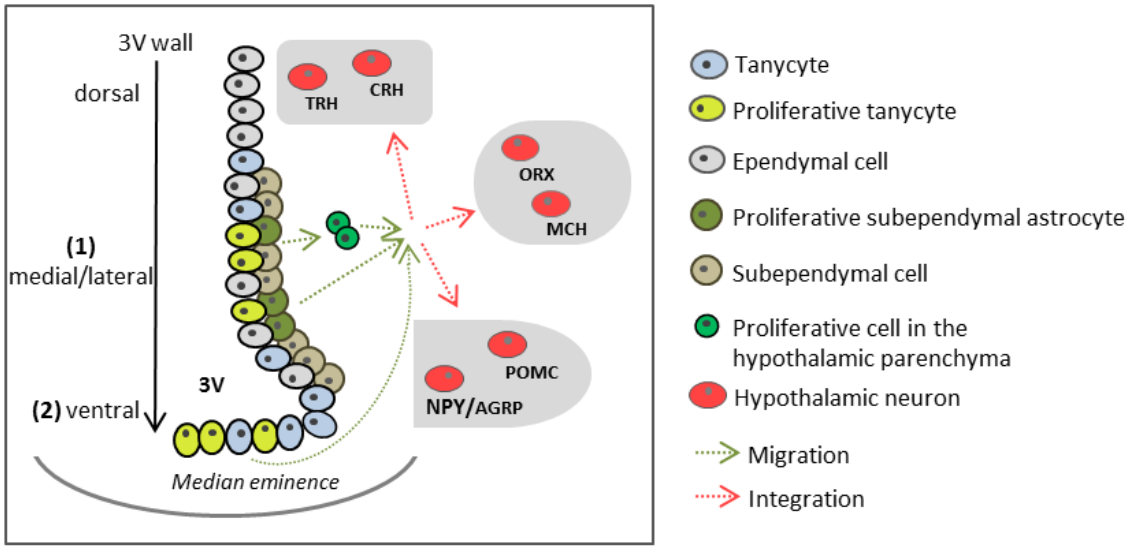

Figure 1.3 – Representation of the hypothalamic neurogenic niche in rodents. Two niches have been appointed (1) in the lateral wall of the 3V and (2) in the ventral wall (median eminence) of the 3V. The 3V wall is constituted of ependymal cells, subependymal cells and tanycytes. The proliferative capable cells proliferate and migrate to the hypothalamic parenchyma and may then differentiate into hypothalamic neurons and be integrated. Adapted from Sousa-Ferreira, *et al*., 2014.

For the evaluation of neurogenesis and identification of hypothalamic NPCs different markers may be used, as described in **Table 1.1**.

Table 1.1 – Markers for evaluation of neurogenesis and identification of NSC's (Neural Stem Cells) and NPC's (Neural Progenitor Cells) pluripotenty.

Marker	Indicator of	Role	Ref
PCNA	Proliferation	- Replication and DNA repair - Expressed in all cell cycle higher during G1 and S phase	[33, 40]
SOX2	NSC's/NPC's	- Pluripotency - Maintenance of NPC's	[33, 40, 41]
Mash1	NPC's	- Differentiation of NSC's to NPC's	[33, 40]
Musashi1	NSC's/NPC's	- Represses proliferative proteins - Necessary for proliferation capacity	[42]
Notch1	NSC's/NPC's	- Maintenance of NSC's quiescent state - Expressed in NPC's	[33, 40]

NSC's – Neural Stem cells; NPC's – Neural Progenitor cells

1.3.3 Hypothalamic neurogenesis involvement in feeding regulation

Several evidence support the role of neurogenesis in feeding behavior and body homeostasis [35]. Hypothalamic cell proliferation studies showed important evidence of a neurogenic activity like proliferation, migration and differentiation of newborn cells [34, 35]. In detail, it was shown that hypothalamic NPC's have the ability to proliferate upon different stimuli such as lesions, hormones and nutritional state [34]. Importantly, newborn cells migrate from the 3V wall to the hypothalamic parenchyma and integrate in pre-existing circuits [34]. Moreover, hypothalamic NPC's can give rise to different neuronal types including neurons involved in feeding regulation (POMC, NPY and orexin positive neurons) and glia cells [35].

Neurotrophic factors including BDNF, insulin growth factor 1 (IGF-1) and ciliary neurotrophic factor (CNTF) promote cell proliferation and maturation in the brain [34] and they are also associated to hypothalamic physiology. BDNF is expressed in the VMH and PVN [19] and its expression can be stimulated by leptin and insulin [34]. Moreover, mice with reduced BDNF signaling have increased food intake and body weight [18]. Regarding neurogenesis activity, BDNF intracereboventricular (i.c.v) infusion in the lateral ventricle showed the capacity to trigger proliferation and generates new neurons [43]. IGF-1 is widely expressed in peripheral organs and in the brain, including the hypothalamus, and is involved in the regulation of endocrine system [44]. IGF-1 stimulates hypothalamic neurogenesis [39]. CNTF is expressed in glia cells, usually related to injury situations and, in the hypothalamus, is expressed by tanycytes and ependymal cells [34, 45, 46]. Apart from promoting neurogenesis, CNTF activates the phosphorylation of STAT3 (signal transducer and activator of transcription 3) similarly to leptin, affecting the feeding behavior and energy availability [34, 45, 46].

1.3.4 Hypothalamic Neurogenesis and obesity

Neurogenesis is a highly regulated process that is sensitive to the organism conditions, consequently it can be altered regarding the body homeostatic state [37, 47]. However, how neurogenesis and obesity are linked and how it affects the body homeostasis regulation is an important issue.

Modifications of neuronal development due to maternal diet are a good example of the relation between homeostasis and neurogenesis [20]. Pre-natal nutrition state modifies the feeding circuits as well as the activity of the sympathetic autonomic nervous system in the adult [20]. In fact, if the mothers are exposed to over-nutrition or nutrient restriction, the offspring presents a higher number of hypothalamic orexigenic neurons [20]. Moreover, animals exposed to maternal over nutrition present leptin resistance and predisposition to become obese in adulthood [20].

Regarding adult neurogenesis in obesity condition, it has been appointed to influence weight regulation and metabolic activity and alter the outcome of obesity [37, 46]. For example, the inhibition of MEm neurogenic activity by radiation targeting led to a reduction of body weight and fat mass when compared to non-irradiated controls in rodents exposed to HFD for 75 days [37]. Additionally, the oxygen consumption and energy expenditure were higher in these animals [37].

In contrast, the administration of CNTF stimulated neurogenesis in the lateral hypothalamic ventricle zone in mice exposed to HFD for 2 months and also led to a reduction of body weight gain [46]. This effect persisted for some time after treatment termination by activation of a leptin dependent pathway since leptin deficient rodents recover body weight immediately after treatment cessation [46].

The contradictory results concerning hypothalamic neurogenesis and obesity reported in these studies may be explained by the different neurogenic zones that are

involved, as well as for differences on diet composition, duration of HFD regime and time-point of neurogenesis assessment.

Regarding regulatory mechanisms it was observed a reduction of NPC's pool in the hypothalamus of obese rodents [48]. Obesity appears to reduce the proliferative capacity of cells but without affecting differentiation and cell fate [48]. Similarly, there was high apoptosis of newly born neurons in HFD rodents and maintenance of old neurons [48]. This last process may occur to compensate for the loss of newly born neurons and of proliferative capacity. Interestingly this process seems to be reversible upon calorie restriction since it was observed an increase of proliferative cells, when mice previously in HFD were placed in 70% calorie restriction for 4 weeks [48].

In long term HFD was observed differentiation impairment, that were observed to be due to IKKβ/NF-κB overactivation that leads to up-regulation of pro-apoptotic genes, and furthermore up-regulation of Notch thus inhibiting differentiation [30].

A study with mutant mice presenting AgRP neurons loss showed that adult neurogenesis may overcome neuronal loss in the hypothalamus [49]. Upon AgRP neurons loss, proliferative mechanisms are activated and afterwards can lead to a population of new neurons [49]. Moreover, these neurons gain of function was observed by leptin responsiveness [49].

1.4 MicroRNAS

MicroRNAs (miRNAS, miRs) are single stranded non coding molecules that regulate gene expression by interacting with mRNAs, as a post-transcriptional mechanism [50-55]. These molecules are tissue specific [53, 54], highly conserved, highly stable and may have more than one target [55]. Also, they are key players in very important processes, such as proliferation, differentiation, growth and development, cell death and metabolism [56]. Deregulation of microRNA pathway has been observed in numerous diseases, including cancer and metabolic diseases [54].

The processing of miR occurs in the nucleus and the cytoplasm (Figure 1.4). The primary miRNA (pri-miRNA) that contains an hairpin with the mature form of the miRNA [57], is transcribed from different genes by RNA polymerase II forming a double-stranded pri-miRNA. miRNAs are than processed in the nucleus by Drosha coupled to DGCR8 (DiGeorge syndrome chromosomal region 8), a RNA type III endonuclease [57, 58], resulting in a double stranded pre-miRNA. DGCR8 function is to act as molecular anchor to properly position Drosha's catalytic site [52]. This pre-miRNA is transported to the cytoplasm by exportin 5 and it is then processed by RNase III enzyme Dicer, coupled to TRBP (trans-activation response RNA-binding protein), producing a double stranded miRNA form. The double stranded miRNA then suffers unwinding and it is transferred to Ago2 (Argonaute2), that belongs to the complex Dicer/TRBP [52]. The mature single stranded miR is loaded into the miRNA-induced Silencing Complex (miRISC). The miRNA along with various proteins in the miRISC identify and regulate the target mRNAs [52]. This occurs by binding of specific complementary base pairs. With high levels of complementarity there is cleavage of the target mRNAS [57, 59]. In an imperfect paring it occurs suppression of translation [57, 59] that implies more miRISC associating molecules with deadenylation role, such as

PABP (Poly-adenylated binding protein), and decapping, like Ddx6 (DEAD (Asp-Glu-Ala-Asp) box helicase 6) of mRNAs [52]. Regarding the regulation of miR biogenesis, it was observed that a target mRNA and the miRNA are able to regulate each other [52].

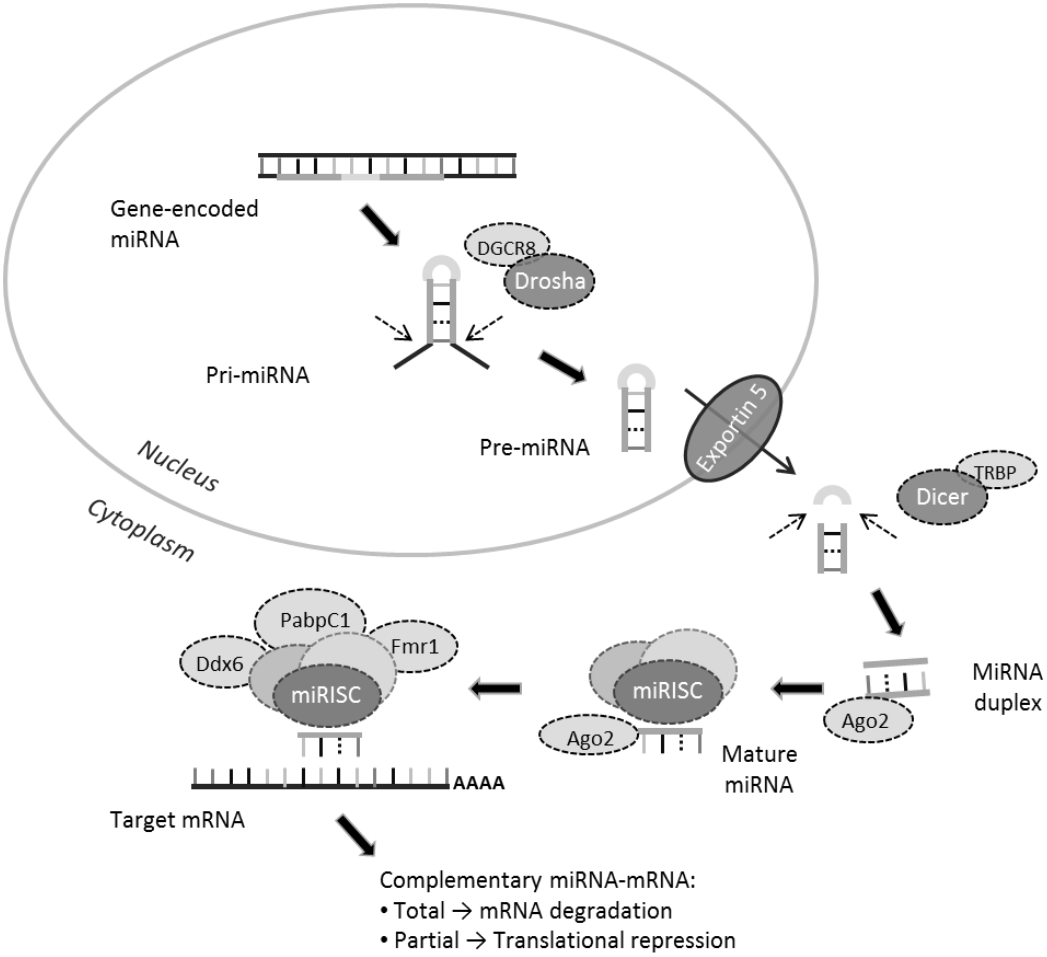

Figure 1.4 - MiRNA biogenesis process. Transcription and processing in the nucleus by Drosha coupled to DGCR8 leads to a pre-miRNA that is transported to the cytoplasm by Exportin 5. In the cytoplasm pre-miRNA is processed by Dicer coupled to TRBP into a double stranded mature miRNA. Ago2 integrates the mature miRNA in the miRISC, where various proteins are present, such as Ddx6, PabpC1 and Fmr1. In the miRISC, miRNA binds to the target mRNA, by complementarity, and inhibits its processing. This can be by cleavage of the mRNA or by translational repression. Adapted from Sousa-Ferreira *et al.*, 2014.

1.4.1 MiRNAs and Metabolism

Several studies have taken an interest in the regulatory role of microRNAs in metabolism. Since microRNAS are highly regulated molecules dysfunction of their part indicates cellular and physiological alterations [60]. However the role of numerous microRNAs have not been described, they regulate different cellular functions, including metabolic processes such as adipocyte differentiation and insulin, lipids and glucose metabolism [51, 60, 61].

1.4.2 Role of miRNAs in adipogenesis

MiRNAs can regulate adipogenesis [51, 61] and the miR upregulated during adipocyte differentiation (such as miR-103, miR-107 and miR-143) are diminished in adipocytes from obese mice [62].

MiR-103 particularly is involved in the regulation of transcriptions factors, that regulate adipocyte differentiation and proteins associated with lipid and glucose metabolism [62]. A study showed that miR-103, among others like let-7b, are involved in the first stages of pre-adipocyte differentiation while miR-103 is upregulated further on when there is formation of lipid droplets [63].

Moreover, the miR expression differential pattern in adipogenesis and obesity were mimicked, though to a minor extent, when 3T3-L1 cells were treated with TNF-α, a cytokine associated with obesity [62]. These results, propose that the changes of miR expression in obesity may be due to inflammation [62].

1.4.3 Role of miRNAs in insulin resistance

Several miRNAs are identified to regulate glucose homeostasis in different tissues including pancreatic β-cells, adipose tissue and liver [51, 61, 64-66].

Pancreatic β-cells produce insulin that reaches target tissues such as skeletal muscle, adipose tissue and liver for glucose metabolism. In hyperglycemia state the production of insulin by pancreatic β-cells is not enough to maintain homeostasis, since insulin signaling is impaired; it results in insulin resistance in advanced situations death of pancreatic β-cells.

Under hyperglycemic conditions, miR-9, in the pancreatic β-cells and miR-29, expressed in the adipose tissue, liver, kidney and muscle, are upregulated; these miRs are involved in insulin release which can lead to insulin resistance [51, 65].

In the adipose tissue, miR-222, miR-29a, miR-27a upregulation is associated with hypoglycemic state, these are predicted targets for MAPK (Mitogen-activated protein kinase) and insulin signaling pathways [64].

In the pancreatic islet β cells, miR-124a overexpression leads to a decrease in insulin secretion [65, 67]. In high glucose concentrations, miR-30d is upregulated and increases insulin gene expression [61, 65, 68]. Also in the pancreas, miR-375, is highly abundant and its overexpression enhances insulin secretion having a key role in blood glucose homeostasis regulation [65, 69]. As for let-7b, it is highly abundant in the pancreatic islet cells [61] and up-regulation may lead to insulin resistance, being a regulator of glucose metabolism related genes [70].

1.4.4 MiRNAs and the hypothalamus

The homeostatic regulation of the central nervous system relies on the hypothalamus [10, 15] and the expression of miRs in this region is involved in the regulation of appetite and metabolic function [55, 61].

In the mouse hypothalamus, among the most expressed miRs are miR-7a and b, miR-124a, miR-125a, miR-136, miR-212, miR-138, miR-338, miR-451 and let-7c [71]. In a different study it was analyzed the hypothalamic ARC and PVN of rats and it was concluded that the same miRNAs are expressed but at different levels [72]. In the rat hypothalamus, the more expressed miRs are let-7c, miR-7a and miR-9 [72].

Interestingly, we can observe an overlap between miRs expressed in the hypothalamus and periphery tissues involved in the glucose metabolism, such as miR-124a2 and miR-9 [61].

Since hypothalamus has an important role in feeding regulation and body homeostasis, it is interesting to observe that there is a differential expression of microRNAs in the hypothalamus dependent of the diet regime. A study analysed miRNA profile in the hypothalamus of rats in a chow diet, HFD and caloric restriction for 3 months [73]. They observed de-regulation of 74 out of 326 expressed in the hypothalamus dependent of caloric intake. They focused on 7 microRNAs since their family's showed the greater alterations, miR-30e, let-7a, miR-9, miR132, miR-145, miR-218, miR-200 [73]. Additionally these microRNAs expression was modified according to time of caloric regime, furthermore they were predicted to have a role in neurogenesis, inflammatory pathways, leptin and insulin resistance [73].

In a anorexia mouse model, anx/anx mice, characterized among other things by a decreased food intake and impairment in the expression of some neuropeptides involved

in feeding regulation [74], was observed an up-regulation of miRISC complex genes in the hypothalamus, indicating changes in the miRNA machinery [74].

In another study, blocking leptin with an antagonist in early life led to increased body weight gain and insulin resistance which was associated to changes in hypothalamic miRNA expression [75].

Regarding leptin, analysis of hypothalamic microRNAs expressed in ob/ob mice (alteration of leptin) and db/db mice (alteration of leptin signalling) showed overexpression of miR-200a which targets leptin [76]. Indeed, miR-200a anti-miR i.c.v infusion in ob/ob mice showed increase of leptin, insulin receptors and IRS2 (Insulin Receptor Substrate-2) expression leading to amelioration of glucose tolerance and re-established liver insulin sensitivity [76].

Dicer expression in the hypothalamus is involved in appetite regulation [77]. In fasting conditions Dicer mRNA is upregulated, and in HFD animals or leptin deficient (ob/ob) mice Dicer mRNA is downregulated [78]. Moreover, Dicer is crucial for POMC neuron survival since Dicer knock-out resulted in POMC neuronal loss, and led to increased adiposity and alterations in glucose metabolism [77].

Furthermore, Dicer knock-out in the ARC of mice resulted in development of obesity. In these mice the loss of Dicer leads to a substantial decrease of a particular microRNA, miR-103 in the ARC, which results in hyperphagya and obesity. Furthermore, injection of a miR-103 mimic revealed reduction of body weight and food intake [79].

Despite all these studies, the role of specific hypothalamic miRNAs in feeding regulation and obesity is still largely unknown and it may lead us to a broader knowledge of the feeding regulation and body homeostasis.

1.4.5 MiRNAs and neurogenesis

Adult neurogenesis is regulated by miRNAs [80]. MiRNAs are required for both maintenance of the neurogenic niche and for differentiation and migration of mature cells [80]. A study showed that deletion of Dicer, impairing miRNA function in neural stem cells, increases the expression of apoptotic proteins and decreases the levels of survival proteins, causing a unbalance of the cell survival/cell death ratio [81].

Neurogenesis is a very tuned regulated process and miRNAs being able to regulate various functions of the cell are key regulators of neurogenesis. For example, let-7 expression is increased during differentiation and is involved in neuronal commitment [80, 82]. As for miR-124 is highly expressed in the brain and influences neuronal commitment and miR-9 is involved in both neural differentiation and migration [80, 82]. Interestingly, some miRNAs responsible for neuronal commitment are also expressed in peripheral cells, where they have a regulatory role regarding metabolism, such as the case of let-7, miR-124 and miR-9 [61].

1.4.6 Let-7/Lin28 system

Let-7 miRs are highly conserved through species [57] and have a key role in development, neurogenesis and metabolism [57] (Figure 1.5).

Let-7 microRNAs family is constituted of 9 members with the same seed region, presenting different isoforms with slight differences in the sequences. Rats express 7 isoforms of let-7 family, mice express 10 isoforms and in humans there are 8 isoforms [57]. Let-7 microRNAs are regulated by Lin28 protein, that presents two isoforms, Lin28a and Lin28b [57, 83]. These present similar structures that are present in the nucleus and in the cytoplasm and both regulate let-7 [84]. Regarding let-7 regulation,

affinity assays showed that Lin28 binds to the terminal loop region present both in pri-miR and pre-miR and has a high affinity for let-7, therefore preventing further processing [85].

Lin28 regulates the translation of various proliferative proteins, maintaining the pluripotency capacity and thus have the opposite effect of let-7 [84, 86, 87]. Lin28 is involved in the development, differentiation and pluripotency, much like let-7 [57, 83, 84, 86] (Figure 1.5). In adult rats Lin28b mRNA expression levels are higher in the testis and placenta, followed by the hypothalamus and it has a weaker expression on skeletal and cardiac muscle [88].

In early development, neurogenic and proliferative factors regulate this system expression [83], such is the case of Musashi1 which increases Lin28 levels [89] that in turn decreases let-7 levels, preventing differentiation [57, 85, 86].

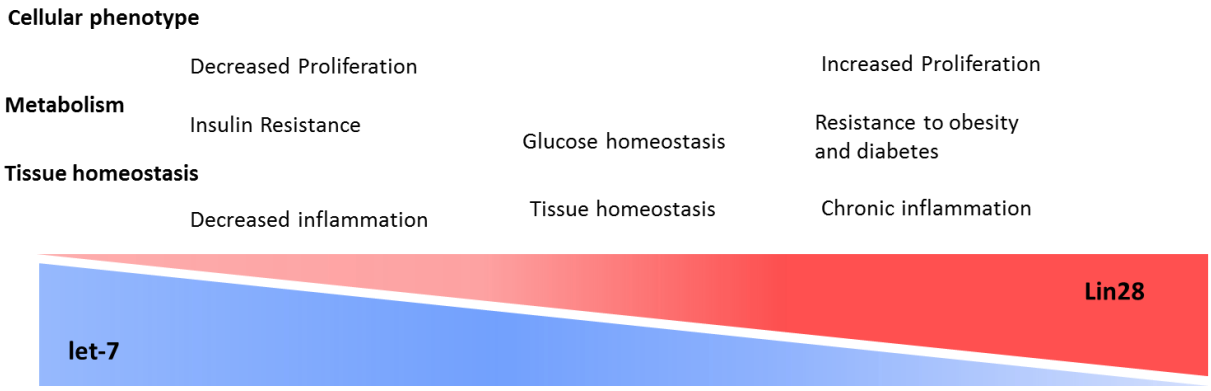

Figure 1.5 – let-7/Lin28 system regulates several functions. The let-7/Lin28 system regulates cellular phenotype, metabolism and tissue homeostasis. High levels of let-7 result in decreased proliferation, insulin resistance and decreased inflammation. As for Lin28 high levels lead to increase proliferation, resistance to obesity and chronic inflammation. Tissue and metabolism homeostasis is reached when the system is regulated. Adapted from Thornton *et al*., 2012.

1.4.7 Let-7/Lin28 and metabolism

Let-7 microRNAs have an important role in metabolism and it is expressed in adipose tissue [63, 90] and pancreatic islet cells [61], as well as in the hypothalamus [72].

Among let-7 isoforms let-7a, let-7b, and let-7d are up-regulated during adipogenesis as shown in *in vitro* study with adipocyte cell line 3T3-L1 [90]. Particularly, let-7b is highly expressed in pre-adipocytes [63], prior to the other isoforms [90]. Regarding let-7a miRNA regulate the change from clonal expansion to terminal differentiation but if added prior to clonal expansion, the process was inhibited [90]; this regulation was observed in a *C. elegans* model in the larva-adult transition [90]. The authors observe that let-7a targets and represses HMGA2 (high-mobility group AT-hook 2), a factor that alters chromatin structure, and it has already been related with low adipose tissue if down-regulated [90].

The peripheral overexpression of let-7a, let-7d and let-7f in mice in a chow regime, resulted in reduction of body weight and fat mass and altered glucose sensing in pancreatic β-cells [91]. Moreover, also in chow fed mice, CNS overexpression of let-7 did not show impairment to glucose tolerance [91]. In another experiment, let-7 was inhibited with an anti-miR injected once a week for the seven weeks of HFD regime [91]. The treated animals presented slower increase of fat mass, normal response to glucose, and improved insulin sensitivity compared to control mice [91]. These results depend only of peripheral response because anti-miRs do not cross the BBB [91].

In another study global overexpression of let-7g led to reduced body size and growth [70]. Regarding metabolic function both chow and HFD fed mice presented glucose intolerance despite producing more insulin [70]. On the other hand, overexpression of Lin28a in the muscle, skin, and connective tissue, resulted in

improved glucose tolerance and insulin sensitivity upon HFD regime [70]. The same occurred in mice with an inducible copy of human Lin28b (gut, spleen, liver and muscle), and additionally mice presented resistance to body weight gain upon HFD [70].

Interestingly, when they crossed the Lin28b-overexpressing mice and let-7g overexpressing mice, no glucose metabolic alterations were observed stating the importance for metabolic function of let-7/Lin28 interaction [70]. To further corroborate this, in Lin28 transgenic mice was observed that let-7/Lin28 system regulates insulin-PI3K-mTOR signaling pathway at different steps and this system alters the expression of related proteins, such as IGFR1, INSR, IRS2, resulting in the alteration of insulin sensitivity and glucose metabolism [70].

In conclusion, let-7 microRNAs are related with glucose metabolism in peripheral tissues however the role of this microRNA in the central regulator of homeostasis (hypothalamus) it is still unknown.

1.4.8 Let-7/Lin28 system and hypothalamus

The hypothalamus is involved in the onset of puberty, which involves the maturation of developmental features, such as brain sexual differentiation. It was observed that to occur maturation let-7 miRs have to be upregulated in the hypothalamus, thus allowing sexual differentiation of the brain [88]. In the neonatal state it was observed high levels of Lin28 protein in the mice hypothalamus [88]. Moreover, the levels of Lin28b diminishes through time reaching the lowest levels in puberty [88].

Further supporting these evidence, alterations in the let-7/Lin28 system in the hypothalamus were observed in animals with delayed puberty [88].

Interestingly, one of the models of delayed puberty used implies a 30% reduction of calorie intake during lactation, which caused a de-regulation of Lin28b and let-7 levels, and suggests that the energy availability influences the let-7/Lin28 system in rat hypothalamus [88].

1.4.9 Let-7/Lin28 system and neurogenesis

Lin28 protein maintains cells proliferative ability [83, 86] and interacts with embryonic stem cells transcription factors , such as Musashi1 [89], IGF, HMGA, OCT4 [70, 83, 86], SOX2 [83, 86, 87] and BDNF [83] causing the inhibition of let-7 [83, 86].

Musashi1 (MS1) is an mRNA binding protein greatly expressed in embryonic stem cells, and binds Lin28 protein [89]. It interacts with Lin28 both in the nucleus (RNA-independent manner) and in the cytoplasm, influencing let-7 regulation [89]. Concerning let-7, MS1 knocked-down induced an increase in Lin28 cytoplasmic expression, and no significant alterations in let-7 expression [89]. But, as expected, the knocked down of Lin28 caused a significant increase of let-7 expression [89]. However the increase was even higher when both Lin28 and MS1 were knocked down in embryonic stem cells [89]. This shows a synergetic effect of Lin28 and MS1 interaction on the regulation of proliferation/differentiation through let-7 [89].

BDNF has a key role in neuronal differentiation, survival, and synapse structure and function. BDNF induces Lin28 expression to repress let-7 target genes, such as Dicer causing an up-regulation of all Dicer-dependent miRs [92]. When BDNF levels decrease, Lin28 decreases and Dicer levels also decrease, due to let-7 release and the levels are maintained upon differentiation [92].

Another important factor in neurogenesis and proliferation of neural precursors is SOX2, which regulates Lin28 and consequently let-7 expression levels [87]. The knock-

down of SOX2 in human embryonic stem cells resulted in overexpression of let-7 miRs, particularly let-7b and let-7i, and was overcome with Lin28 overexpression [87]. Furthermore, Mash1, proneural gene is a target of let-7i [87]. It was observed with the overexpression of let-7i and the knock-down of SOX2 a reduced expression of Mash1 [87].

1.4.10 Let-7/Lin28 system and inflammation

It was observed a feedback loop involving let-7/Lin28 system and the regulation of pro-inflammatory factors. A study shows that after triggering inflammation, NF-κB levels rose accompanied by an increase of Lin28b and IL6 and a contrast decrease of let-7 miRNA [93]. Moreover, IL6 is targeted by let-7, by direct binding, as well as by interacting with Ras, a known target of let-7 and a NF-κB activator [93]. All in all, there is a negative feedback loop between let-7 and inflammatory factors, and a positive feedback loop between these and Lin28 [93].

1.5 Objectives and Hypothesis

MicroRNAs have important regulatory functions and their expression levels are altered in different pathologies showing their role in disease development. However, microRNAs function in the hypothalamus is largely unknown and their involvement in obesity-induced metabolic and neuropathological alterations has not been investigated yet.

A previous study from our laboratory revealed alterations in let-7 microRNAs expression levels in the hypothalamus of rats in HFD regime. Let-7 is involved in several roles, including metabolism, neurogenesis and inflammation, leading to a particular interest to this miRNA in an obesity context.

Therefore, in this project we will investigate the impact of obesity on hypothalamic microRNA pathway and explore the putative protective effect of let-7b in obesity associated metabolic alterations and neuropathological dysfunction.

In detail, this project includes three specific aims:

- To analyse how HFD consumption affects hypothalamic microRNA pathway and, in particular, the expression levels of let-7 microRNAs and miRISC genes. In this experiment, different periods of HFD consumption will be evaluated, since there are differences between short and long term HFD in processes such as neurogenesis and inflammation.
- To identify the mechanism responsible for miRNA alterations in the hypothalamus. For this, we will induce inflammation in hypothalamic slice cultures and analyse let-7 expression levels to find if it mimics HFD alterations.
- To investigate let-7 microRNA as a possible candidate for an anti-obesity role, and understand the mechanism(s) of its putative protective effect. In order to achieve this, we will use lentiviral vector technology to overexpress let-7b in the

mice hypothalamus. Mice will be fed with an HFD and we will evaluate metabolic parameters (body weight, food intake, glucose metabolism) and neuropathological dysfunction in their hypothalamus (neurogenesis, inflammation, insulin signaling).

On an overall, we hypothesize that let-7 microRNAs can prevent metabolic alterations induced by obesity by inhibiting hypothalamic neuropathological alterations occurring upon HFD consumption. In this way, we expect to identify let-7 microRNA as a new candidate for obesity therapeutics.

Chapter II. Materials and Methods

In vivo experiments

All experimental procedures were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. In addition, animals were housed in a licensed animal facility (international Animal Welfare Assurance number 520.000.000.2006) and the CNC animal experimentation board approved the utilization of animals for this project. Moreover, people coordinating the animals studies have received appropriate education (FELASA course) as required by the Portuguese authorities.

2.1. Experiment 1 –MiRNAs and neurogenesis in high fat diet exposed rats

2.1.1 Animals

Male Wistar rats were purchased from Charles River Laboratories and randomly divided into three groups. Rats were housed under a 12h light/dark cycle in a temperature/humidity controlled room with *ad libitum* access to water and food. Control rats were maintained in a normal chow diet (8% fat) and high-fat diet rats were maintained in a 40% fat diet (LabDiet – Western diet for rodents), for 4 or 8 weeks. Body weight was measured weekly, food intake was evaluated three times (weeks 2, 4 and 8) and glycemia was monitored two times (weeks 4 and 8) using the FreeStyle Precision Neo glucometer (Abbot).

2.1.2 Tissue collection

Rats were sacrificed with a lethal dose of anesthetic (Pentobarbital), followed by decapitation. Brains were removed; the hypothalamus was dissected to a depth that the

entire ventral and dorsal nuclei were removed; and cortex samples were dissected. Tissue samples were immediately frozen in dry ice and kept at -80° C.

2.2. Experiment 2 - Organotypic slice cultures

Hypothalamic slices were obtained from Wistar rats with 7 to 8 days post-natal. Animals were euthanized by decapitation and the brain was dissected, placed in a Petri dish with dissecting medium (PBS solution with 5.5 mM glucose (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Gibco)), and the hypothalamus was isolated.

Hypothalamic slices of 350 µm of thickness were obtained with a tissue chopper (McIlwai Tissue chopper MTC/2). Hypothalamic slices were then separated in dissection medium. In the flow chamber, hypothalamic slices were moved into a new Petri dish and the dissection medium was changed three times. Hypothalamic slices were then moved to cell culture inserts (Millicell cell culture insert, 30 mm diameter of filter, hydrophilic PTFE, 0.4µm, from Milipore) placed in a 6-well multiwell culture plate with 1.4 ml of MEM (minimum essential medium) culture medium supplemented with 25% (v/v) HBSS (Hank's balanced salt solution), 25% (v/v) Horse serum, 35 mM glucose, 5.5 mM L–Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco)). Slices were maintained in an incubator at 37ºC with controlled CO_2 levels. The medium was changed every other day for a total of 7 days.

Lipopolysaccharide (LPS; Sigma) was reconstituted in water. LPS incubation (final concentration 1µg/mL) was performed at different time points: 1h, 4h, 16h, 24h and 48h before tissue collection. Culture medium and tissue were collected for total RNA extraction.

2.3. Experiment 3 – Overexpression of let-7b in high fat diet exposed mice

2.3.1. Animals

Adult male C57BL/6 mice were purchased from Charles River Laboratories and randomly divided into four groups. Mice were housed under a 12h light/dark cycle in a temperature/humidity controlled room with *ad libitum* access to water and food.

A control group was maintained in normal chow diet (8% fat). The animals of the other 3 groups were maintained in a high fat diet (LabDiet - Western diet for rodents) with 40% fat. Body weight gain and food consumption were measured twice a week for a total of 7 weeks.

2.3.2. Plasmid construction and validation

The let-7b precursor and the control microRNA (miR-neg), with a random sequence validated not to target any rat, mouse or human gene, were cloned into a lentiviral plasmid. The miR-neg sequence was previously validated by our group [94].

The let-7b precursor or the control microRNA (miR-neg), were inserted downstream of the H1 promoter. The plasmids also included a reporter gene GFP (Green Fluorescent Protein) expressed under the control of PGK (Phosphoglycerate Kinase 1) Promoter [95]. Let-7b over-expression plasmid was validated by transfection of the plasmid in HEK 293 cell line and quantification of let-7b levels by qRT-PCR (as described in section 2.6.2.1).

2.3.3. Viral production - Lentiviral Vectors

Lentiviral particles were produced in HEK 293T cells using a four plasmid combination, as described before [95]. Afterwards, lentiviral particles were ressuspended in PBS with 0.5% BSA (Bovine Serum Albumin). Samples concentration was assessed by measuring human immunodeficiency virus 1 (HIV-1) p-24 antigen has described in the RETRO-TEK HIV-1 p24 Antigen ELISA (ZeptoMetrix Corporation). Viral stocks were stored at –80ºC.

2.3.4. Stereotaxic Surgery

Male C57BL/6 mice were anesthetized by intraperitoneal injection of 250 mg of Avertin (Sigma) per kg (body weight) and placed on a stereotaxic frame. The animals were injected with 2.5 µl of lentiviral suspension in each hemisphere. Lentiviral particles were prior diluted in 1% BSA in PBS to comprise 400 ng of p-24 antigen. Injection was performed bilaterally into the medial hypothalamus following the coordinates:1.65 mm posterior to bregma; -0.35 mm/+0.35 mm lateral to bregma and 6.00 mm ventral to the brain surface (the needle was first placed at -6.05 and then lifted). Afterwards the syringe was kept in place for 5 more minutes to prevent backflow.

2.3.5. Glucose Tolerance Test (GTT)

Glucose tolerance test was performed at the sixth week of HFD. Mice were fasted for 12h. The next morning mice were weighted and glycemia levels were measured using the FreeStyle Precision Neo glucometer (Abbot) (Time 0). Then, mice were injected intraperitoneally with 2.0 g/kg of sterile glucose (using a 20% glucose solution in saline

0.9% NaCl). Glycemia levels were measured at 15, 30, 60, 90 and 120 minutes after glucose administration.

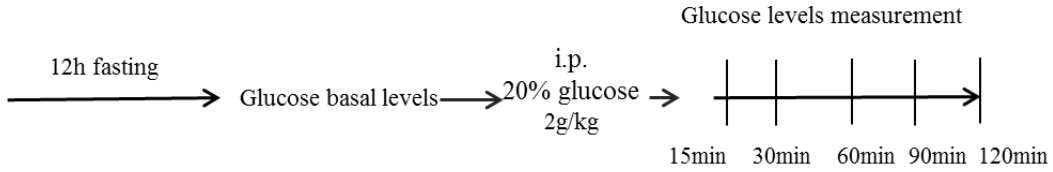

Figure 2.1 – Schematic representation of GTT process. Mice were maintained during 12h in fasting, and glycemia basal levels were measured (time 0). Glucose was administred via i.p. (2g/kg) and glycemia was measured 15, 30, 60, 90 and 120 minutes after glucose injection.

2.3.6. Tissue collection

At week 7, mice were sacrificed with a lethal dose of halothane (2-bromo-2-chloro-1, 1, 1-trifluoroethane) followed by decapitation. The brain, white adipose tissue (epididymal fat pad) and brown adipose tissue (interscapular fat pad) were collected. The hypothalamus was dissected to a depth that the entire ventral and dorsal nuclei were removed. Samples were immediately frozen in dry ice and kept at -80° C.

The white adipose tissue and brown adipose tissue were weighted and, afterwards, samples were collected, immediately frozen in dry ice and kept at -80ºC.

Blood was collected; the serum was separated by centrifugation (2000 g, 15 minutes) and stored at -20^{0}C.

For immunohistochemistry, mice were anesthetized with halothane and perfused intracardially with PBS and 4%paraformaldehyde (PFA) for tissue fixation. Brains were collected, kept at 4^{0}C in 4%PFA overnight, and then in 30% sucrose for 2 days. Afterwards, brains were dried and stored at -80ºC.

2.4. Protein extraction

Protein extracts were obtained from rat hypothalamus and cortex samples by homogenization in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate (SDS)), supplemented with 200 µM phenylmethylsulphonylfluoride (PMSF), 1 mM dithiothreitol (DTT), 1 mM Na3VO4, 10 mM NaF and a complete mini protease inhibitor cocktail tablet, with the help of a pestle, on ice. Samples were then incubated in ice for 30 minutes and afterwards sonicated. Samples were kept at -80ºC.

Before use, samples were thawed, centrifuged for 10 min at 14000 rpm at 4ºC and the supernatant was collected into a fresh 1.5 mL tube.

Protein quantification was performed using the BCA (Bicinchoninic Acid) colorimetric method. Samples were diluted in RIPA buffer (if needed) and denatured with 6x concentrated electrophoresis sample buffer (0.5 M Tris-HCl, 30% glycerol, 10.4% SDS, 0.6 M DTT, 0.02% bromophenol blue; pH 6.8), boiled for 5 min at 95ºC and stored at -20ºC.

2.5. Western Blotting

Western blot technique was performed for detection of neurogenic proteins (Musashi1 and SOX2) and proliferative protein, PCNA. For electrophoresis it was used sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), with 4% stacking gel and 12% resolving gel.

Seventy micrograms of protein were loaded in the same volume (30 µl) for all samples. Additionally, 30 µg of protein of a positive control (embryonic tissue) were also loaded in the gel. A protein marker (Nzytech) was used to determine protein bands size.

Electrophoresis was performed with Tris–Bicine running buffer (25 mM Tris, 25 mM Bicine, 1%SDS (w/v); pH = 8.3) at 70 volts for 10 minutes and 120-130 volts for 70 to 90 minutes.

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane previously activated with methanol, using CAPS buffer (10 mM CAPS, pH= 11.0 with 10% methanol (v/v)) at 4ºC for 3 hours at 750mA. Subsequently, membranes were incubated 1 hour at room temperature in Blocking Buffer (5% (w/v) non–fat milk diluted in TBS-T (Tris-Buffered Saline Tween; 20 mM Tris-base, 0.137 M NaCl and 0.01% Tween)), according to antibody specifications. Next, membranes were washed with TBS-T and incubated overnight with primary antibody at 4ºC.

Afterwards, membranes were washed with TBS-T and incubated with the corresponding secondary antibody, for 1 hour at room temperature. Subsequently membranes were washed, dried with filter paper and placed in contact with ECF (about 500 µl) for 2 to 5 minutes. Proteins were detected in *Versadoc* imaging system (Bio-Rad). Optical density of the bands was quantified with *Quantity One* software.

2.5.1. Antibodies

The primary antibodies used for Western Blot were: anti-rabbit Musashi1 1:1000 (Abcam ab5286), anti-mouse SOX2 1:500 (R&D systems MAB2018); anti-mouse PCNA 1:500 (Santa Cruz sc-56) and for protein loading control it was used anti-mouse Tubulin 1:10 000 (Sigma T7816). All antibodies were diluted in 5% non-fat milk (w/v) in TBS-T. As for the secondary antibodies, anti-rabbit and anti-mouse alkaline phosphatase-linked antibodies (GE healthcare) were used in a 1:10 000 dilution in 5% non-fat milk (w/v) in TBS-T.

2.6. mRNA and microRNA quantification

2.6.1 Total RNA extraction

Total RNA was extracted from the hypothalamus, cortex and hypothalamic slices using the miRCURY RNA Isolation kit for tissue (Exiqon) according to the manufacturer's manual. DNAse digestion was performed during the process to exclude any contamination with genomic DNA. Total RNA was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific) and the purity was assessed with the ratio of OD at 260 and 280 nm. Total RNA samples were kept at -80°C.

2.6.2 qRT-PCR

2.6.2.1 qRT-PCR for microRNAs quantification

Levels of let-7b, let-7c and let-7e microRNAs were assessed by quantitative RT-PCR (qRT-PCR). Briefly, cDNA of miRNAs was produced from the conversion of 5 ng of total RNA using the miRCURY LNA Universal cDNA Synthesis Kit (Exiqon) and according to the manufacturer's protocol. cDNA samples were diluted 40x with RNase-free water and stored at -20°C.

For miRNA quantification, the miRCURY LNA SYBR green master mix was used in combination with pre-designed microRNAs LNA PCR primer sets (both from Exiqon). Reference miRs were miR-103 (Exiqon) for rat samples and SNORD 110 (Exiqon) for mice samples. These reference miRs were previously validated by GeNorm and NormFinder software's.

2.6.2.2 qRT-PCR for mRNA quantification in rat tissues

The mRNA levels of miRISC genes (Ago2, Ddx6, Dgcr8, Fmr1 and PabpC1), inflammatory genes (IL1β, IL6 and IKBK) and Lin28b were assessed by qRT-PCR, as previously described [96]. Briefly, cDNA was obtained from the conversion of 500 ng of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

Prior to use, samples were diluted in RNase-free water and stored at 4°C (final cDNA amount per PCR reaction in Table 1 (A and B). For mRNA quantification, the SsoAdvanced SYBR Green Supermix (BioRad) was used in combination with pre-designed Primers (QIAGEN). The reference gene rat HPRT (QIAGEN) was previously validated by GeNorm and NormFinder software's.

Table 2.1 – Rat genes quantified by qRT-PCR in rat hypothalamus and cortex (**A**) and in rat hypothalamic slice cultures, (**B**). Identification of accession number, final CDNA amount per reaction and annealing temperature for the genes analysed. Primer sequence is not provided by the company (QIAGEN). Primers are provided in 10x concentration and used at 1x final concentration.

A) Rat Hypothalamus and Cortex - Primers and cDNA dilutions

Gene	Accession number	CDNA (ng)	Annealing T^0C
Ago2	NM_021597	5 ng	60^0C
Ddx6	NM_001109292		
Dgcr8	NM_001105865		
Fmr1	NM_052804		
PabpC1	NM_134353		
Lin28b	XM_001069344		
HPRT (REF)	NM_012583		

B) Hypothalamic Slice Cultures (Rat) - Primers and cDNA dilutions

Gene	Accession number	CDNA (ng)	Annealing T^0C
Ago2	NM_021597	2.5 ng	60^0C
Ddx6	NM_001109292	2.5 ng	
Dgcr8	NM_001105865	2.5 ng	
Fmr1	NM_052804	2.5 ng	
PabpC1	NM_134353	0.33 ng	
Lin28b	XM_001069344	50 ng	
IL1β	NM_031512	2.5 ng	
IKBKB	NM_001190720	2.5 ng	
HPRT (REF)	NM_012583	2.5 ng	

2.6.6.3 qRT-PCR for mRNA quantification in mouse tissue

The mRNA levels of miRISC genes (Dicer, Drosha and Exportin 5), Lin28b, neurogenesis markers (BDNF, IGF1, Mash1, Musashi1, NeuroD1, Notch1), proliferation marker (PCNA), hypothalamic neuropeptides (AgRP, NPY, POMC), insulin signaling pathway constituents (InsR, IRS1), leptin receptor (LepR), inflammation mediators (IL1β and IL6) and a gliosis indicator (GFAP) were analyzed in mice hypothalamic samples by qRT-PCR as previously described [96]. Briefly, cDNA was obtained from the conversion of 500 ng of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Prior to use, samples were diluted in RNase-free water and stored at 4°C (final cDNA amount per PCR reaction in Table 2). For mRNA quantification, the SsoAdvanced SYBR Green Supermix (BioRad) was used in combination with pre-designed QuantiTect Primers (QIAGEN) or pre-designed KiCqStart Primers (Sigma). Reference gene HPRT (QIAGEN) was previously validated by GeNorm and NormFinder software's.

Table 2.2 - Mouse genes quantified by qRT-PCR. Identification of accession number, primer sequence, primer dilution, final CDNA amount per reaction and annealing temperature for the genes analysed in the mouse hypothalamus. QIAGEN primers sequence is not provided by the company.

Mouse Hypothalamus - Primers and cDNA dilutions

Gene	Company	Accession number	Primer Sequence (5'-3')	Primer Dilution	cDNA (ng)	Annealing T^0C
Dicer	Qiagen	NM_148948	Not Provided	1X	25 ng	60^0C
Drosha		NM_001130149		1X	25 ng	60^0C
Exportin 5		NM_028198		1X	25 ng	60^0C
Lin28b		NM_001031772		1X	25 ng	60^0C
BDNF		NM_001285416		1X	25 ng	60^0C
IGF1	Sigma	NM_010512	F: GACAAACAAGAAAACGAAGC R: ATTTGGTAGGTGTTTCGATG	500 mM	25 ng	58^0C
Mash1		NM_008553	F: GACTTTGGAAGCAGGATG R:ACATTGCATCTTAGTGAAGG	500 mM	25 ng	60^0C
Musashi1		NM_008629	F: GTTTGGATTTGTCACGTTTG R: TCTTGCATTCCACCATTTTG	500 mM	25 ng	60^0C
NeuroD1		NM_010894	F: AGATCGTCACTATTCAGAACC R: AGAACTGAGACACTCATCTG	500 mM	100 ng	60^0C
Notch1		NM_008714	F: ACGTAGTCCCACCTGCCTAT R:ACAGGTGCCCTGATTATAGC	500 mM	25 ng	56^0C
PCNA		NM_011045	F: GCACGTATAGCCGAGACCT R:TTGCCAAGCTCTCCACTTGC	500 mM	25 ng	56^0C
AgRP	Sigma	NM_007427	F: AGGTCTAAGTCTGAATGGC R: CGGTTCTGTGGATCTAGC	500 mM	25 ng	58^0C
NPY	Qiagen	NM_023456	Not Provided	1X	25 ng	60^0C
POMC		NM_008895	F: AAAAGAGGTTAAGAGCAGTG R: ACATCTATGGAGGTCTGAAG	500 mM	25 ng	58^0C
InsR		NM_001079817	F: AAGACCTTGGTTACCTTCTC R:GGATTAGTGGCATCTGTTTG	500 mM	25 ng	60^0C
Irs1		NM_010570	F: GATCGTCAATAGCGTAACTG R: ATCGTACCATCTACTGAAGAG	500 mM	25 ng	60^0C
LepR		NM_146146	F: CTGAGATACAGTACAGCATTG R: TGATATTGACATCGATCACG	500 mM	100 ng	60^0C
IL1β		NM_008361	Not Provided	1X	100 ng	60^0C
IL6		NM_031168		1X	100 ng	60^0C
GFAP	Sigma	NM_010277	F: GGAAGATCTATGAGGAGGAAG R:CTGCAAACTTAGACCGATAC	500 mM	100 ng	58^0C
HPRT (REF)	Qiagen	NM_013556	Not Provided	500 mM	25 ng	60^0C

2.6.2.3 PCR conditions and analysis

For each reaction, 6 μL of master mix were added to 4 μL of template cDNA (final concentrations in Table 1A, 1B and Table 2). All reactions were performed in duplicate (two cDNA reactions per RNA sample). PCR was performed in a StepOnePlus thermocycler (Applied Biosystems). For mRNAs quantification, the protocol was the following: 95ºC for 5 min; and then 40 cycles, 95ºC for 10 seconds followed by 30 seconds of the specific annealing temperature. For microRNAs quantification, the protocol was the following: 95ºC for 10 seconds; and then 40 cycles, 95ºC for 10 seconds and 60ºC for 1 minute. In both protocols the melting curve protocol started immediately after amplification. PCR runs with RNA samples (no RT samples) served as negative controls.

The amplification efficiency for each target and the threshold values for threshold cycle determination (Ct) were determined automatically by the StepOnePlus software (Applied Biosystems). Relative mRNA or microRNA quantification was performed using the ΔCt method for targets with the same amplification efficiency.

2.7. Statistical Analysis

Results are expressed as mean and SEM.

Body weight gain, food intake and qRT-PCR of the rat experiments were analysed by one-way ANOVA followed by post hoc Tukey test to determine significant differences between chow and HFD groups (4 weeks and 8 weeks). As for the western blot analysis, T-test was used to compare 4 weeks HFD with chow and 8 weeks HFD with chow.

Hypothalamic slice cultures qRT-PCR (mRNA and microRNAs) were analysed by one-way ANOVA followed by post hoc Tukey test to determine significant differences between the different time points analysed 0h (control), 1h, 4h, 16h and 24h.

For the mouse experiments, the cumulative body weight gain is presented in a non-linear regression followed by F-test to determine significant differences between slopes. Epididymal WAT weight presented as percentage of body weight and food intake were analysed by T-test. Glucose Tolerance Test curves were analysed by Two-way ANOVA with post hoc Bonferroni test to determine significant differences between groups. Area under the curve (AUC) data of the glucose tolerance test was analyzed by a T-test. All the qRT-PCRs were analysed by T-test to determine significant differences between the two groups analyzed. For all the statistical tests, $p<0.05$ were considered significantly different.

Chapter III. Results

3.1. High fat diet causes metabolic and hypothalamic alterations in rats

High fat diet (HFD) causes metabolic alterations in rats

In order to evaluate whether HFD consumption results in metabolic alterations in this rat model, body weight and food intake were measured. As expected, rats on HFD regime gained more weight and showed higher caloric intake than rats on regular chow diet (Figure 3.1-**A** and **B**, respectively).

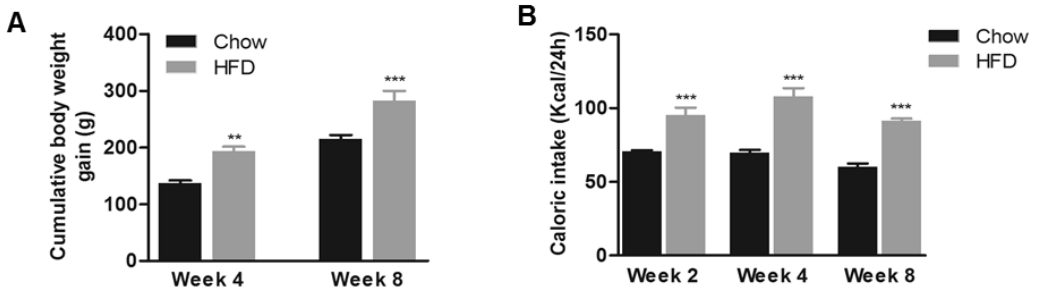

Figure 3.1 - High-fat diet (HFD) induces metabolic alterations. Body weight gain **(A)** and daily caloric intake **(B)** of animals exposed to HFD for 4 weeks and 8 weeks. n=6-12 rats; One-Way ANOVA; ** $p<0.01$, *** $p<0.001$, compared to same week chow.

High-fat diet consumption alters microRNA pathway

To determine whether microRNA pathway is affected by HFD consumption, we assessed the mRNA levels of genes involved in microRNAs biogenesis and activity (miRISC genes) in the hypothalamus and cortex (Figure 3.2). The genes evaluated (Ago2, Ddx6, Dgcr8, Fmr1 and PabpC1) were previously reported as altered in mice models of central nervous system disorders, such as anorexia and Huntington disease [74, 97].

In this study, we found that mRNA levels of Dgcr8, Fmr1 and PabpC1 are down-regulated in the hypothalamus of rats after 4 weeks of HFD and levels of Ago2 and Dgcr8 are down-regulated after 8 weeks of HFD (Figure 3.2-**A**), showing an impairment of the hypothalamic microRNA pathway.

MicroRNA pathway deregulation in the cortex was also explored and it was observed that the mRNA levels of Dgcr8 and Fmr1 are down-regulated after 4 weeks of HFD and mRNA levels of Ddx6, Fmr1 and PabpC1 are upregulated after 8 weeks of HFD in the cortex (Figure 3.2-**B**).

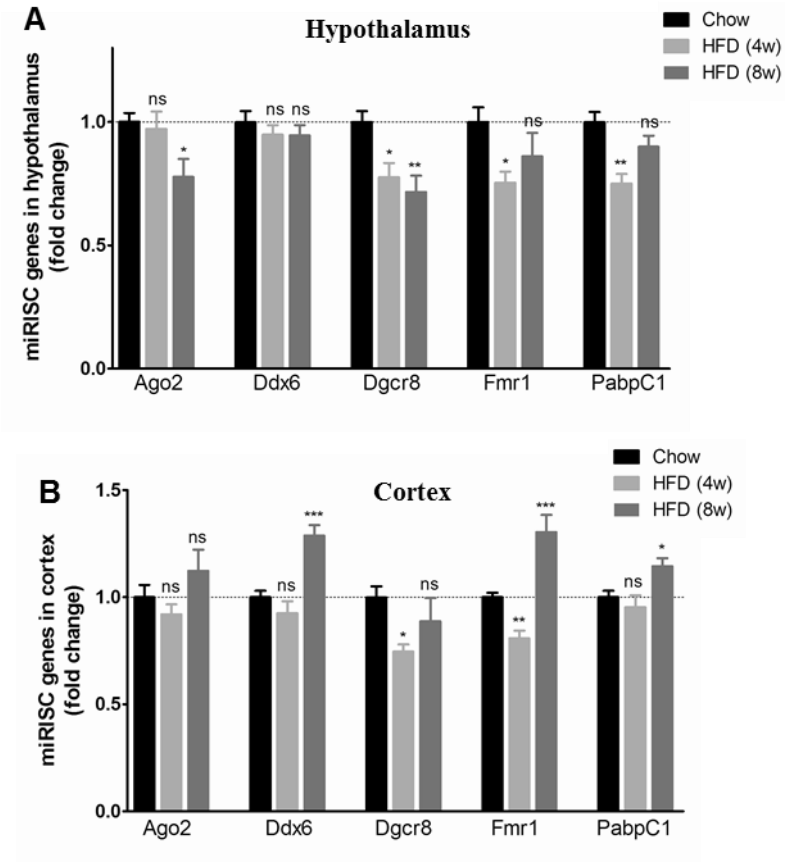

Figure 3.2 – MicroRNA pathway is altered after High-fat diet (HFD) consumption. Changes in mRNA expression levels of miRISC genes, Ago2, Ddx6, Dgcr8, Fmr1, PabpC1 in the rat hypothalamus **(A)** and cortex **(B)** after 4 and 8 weeks of HFD consumption. n=5-12; One-way ANOVA; ns $p>0.05$, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, compared to same gene in chow diet.

HFD consumption causes alteration of let-7/Lin28 system

A microRNA microarray had been previously performed in our laboratory to determine the microRNAs expression profile of the rat hypothalamus. This microRNA microarray showed that, 7 miRNAs to the let-7 family were downregulated in the rat hypothalamus

upon 4 weeks of HFD (data not shown). Moreover, six members of the let-7 family are included in the thirty most expressed microRNAs in the hypothalamus, as determined in the same microarray (data not shown). Let-7 microRNAs are of interest because of their association with neurogenesis, inflammation, insulin sensitivity and glucose metabolism [70, 87, 91, 98].

Therefore, let-7 microRNAs levels were analysed in the rat hypothalamus after 4 and 8 weeks of HFD regime, using qRT-PCR technique. It was observed a decrease of let-7b expression levels at 4 weeks of HFD, followed by an increase at 8 weeks of HFD, in the hypothalamus (Figure 3.3-**A**). Let-7c and let-7e were also increased in the rat hypothalamus after 8 weeks of HFD (Figure 3.3-A). Furthermore, a key protein for the let-7 microRNA maturation, Lin28b was analysed [83]. mRNA expression levels of Lin28b in the hypothalamus increased at 4 weeks HFD and return to control (chow) levels at 8 weeks of HFD (Figure 3.3-**B**). Interestingly, this is a partially reverse expression compared to let-7b miRNA (Figure 3.3-**A**).

Also, analysis of cortex samples showed increased expression of let-7b, let-7c and let-7e in rats in a HFD regime for 8 weeks (Figure 3.3-**C).** But, as for the mRNA levels of Lin28b in the cortex of rats in HFD, no changes were observed when compared to rats in chow diet (Figure 3.3-**D**).

Together, these data show that different periods of HFD alter let-7 miRNAs and related protein Lin28b expression levels in the hypothalamus, suggesting a role of these microRNAs in the hypothalamic response to HFD.

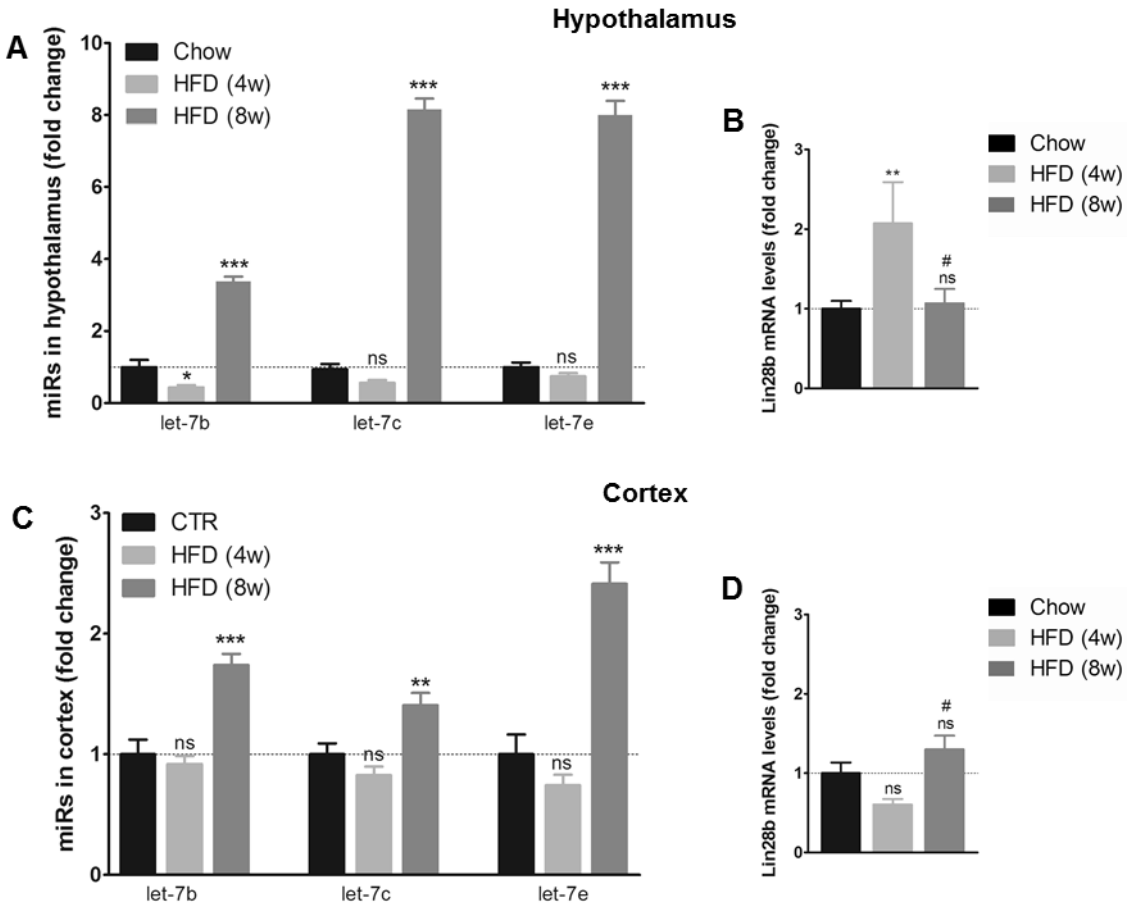

Figure 3.3 – Impact of high-fat diet (HFD) on let-7 microRNAs levels. In rat hypothalamus **(A)** and cortex **(C)**; and, alteration of lin28b mRNA expression levels in rat hypothalamus **(B)** and cortex **(D)** after 4 and 8 weeks of HFD consumption. n=5-12; One-way ANOVA; ns $p>0.05$, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, compared to chow; $^{\#}$ $p<0.05$ compared to HFD (4 weeks).

Alteration of neurogenesis markers after 4 weeks HFD in the hypothalamus

Following the alterations observed in the let-7/Lin28b axis in the hypothalamus of rats in HFD and since this system has an important role in proliferation [83], we evaluated neurogenesis markers levels in the rat hypothalamus by western-blot. It was observed an increase of Musashi1 and SOX2 protein levels (neuroprogenitor cells markers) after 4 weeks of HFD (Figure 3.4-**A** and **C**) that returned to control levels after 8 weeks of HFD (Figure 3.4-**B** and **D**). These data suggest that after a short-term HFD

hypothalamic neurogenesis is activated, but this mechanism is not stimulated in long term of HFD consumption. In contrast, the protein levels of proliferation marker, PCNA were not altered in any of these conditions (Figure 3.4-**A**, **B, C** and **D**).

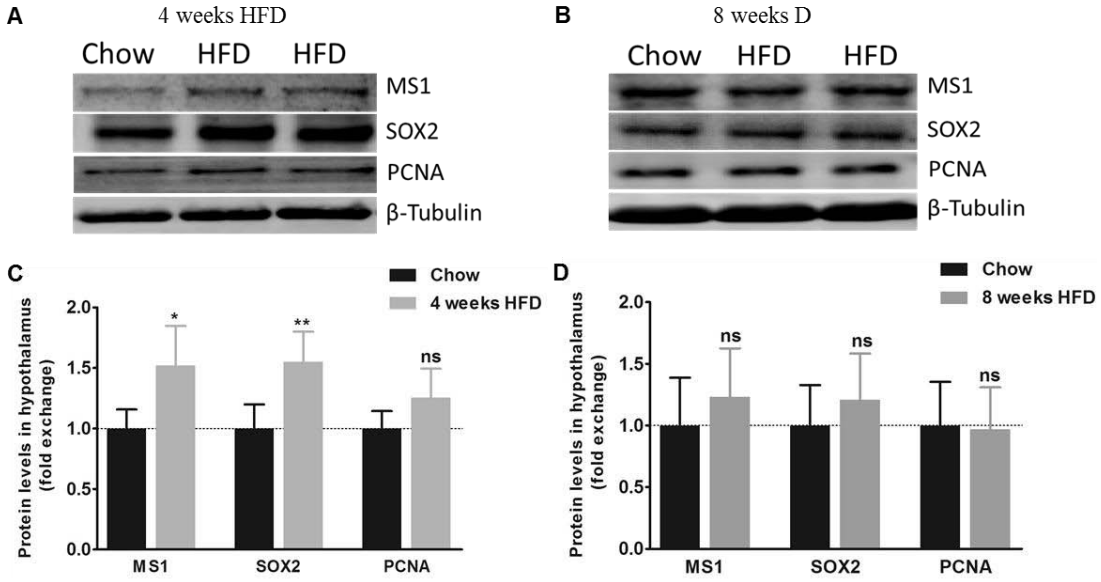

Figure 3.4 – Short-term HFD (4 weeks) increases protein levels of neurogenesis markers (Musashi1 and SOX2) in the rat hypothalamus. Protein levels of Musashi1 (MS1), SOX2 and PCNA in the hypothalamus of rats after 4 weeks **(A** and **C)** and 8 weeks **(B** and **D)** of HFD regime. Band intensity quantification normalized to tubulin. n=5-12, Unpaired T-test; ns $p>0.05$, * $p<0.05$, ** $p<0.01$, compared to chow.

HFD does not alter neurogenesis markers in the cortex

As shown above in Figure 3.3-**C**, apart from the hypothalamus, let-7 microRNAs are altered in the cortex after 8 weeks of HFD. Consequently, we evaluated neurogenesis markers in rat cortex. Western-blot analysis showed that the expression levels of neurogenesis markers are not altered in the cortex after HFD consumption, in either of the time-points analysed (Figure 3.5-**A**, **B**, **C** and **D**). SOX2 protein could not be detected in these samples.

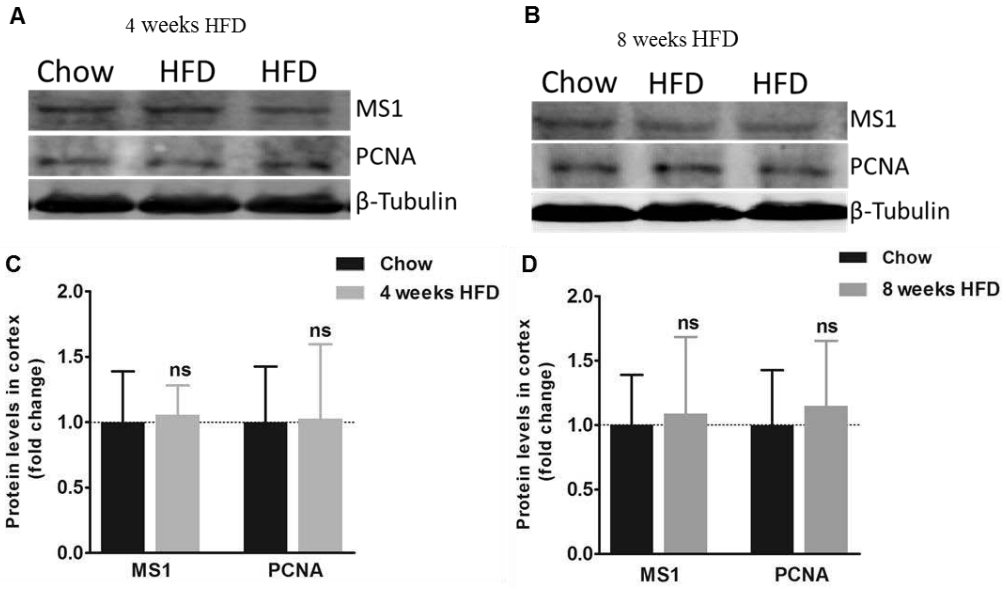

Figure 3.5 - High-fat diet (HFD) consumption does not change neurogenesis markers levels in the rat cortex. Protein levels of neurogenesis markers Musashi1 (MS1) and proliferation marker (PCNA) in the cortex of rats after 4 weeks **(A** and **C)** and 8 weeks **(B** and **D)** of HFD regime. SOX2 protein was not detected in these samples. Band intensity quantification normalized to tubulin. n=5/12, Unpaired T-test, ns $p>0.05$ compared to chow.

3.2. LPS induced inflammation in hypothalamic slices mimic HFD alterations in let-7/Lin28b system

Since HFD induces inflammation in the rodent hypothalamus [9], we investigated whether inflammation alone could cause the alterations in the microRNA pathway observed in the rat hypothalamus in our previous experiment. For that, hypothalamic slice cultures were incubated with LPS, a proven inducer of inflammation, at a concentration of 1µg/mL as previously described [99] for 1h, 4h, 16h, 24h and 48h (Figure 3.6). Organotypic cultures were used in the experiment because they maintain the different cell types present in the hypothalamus.

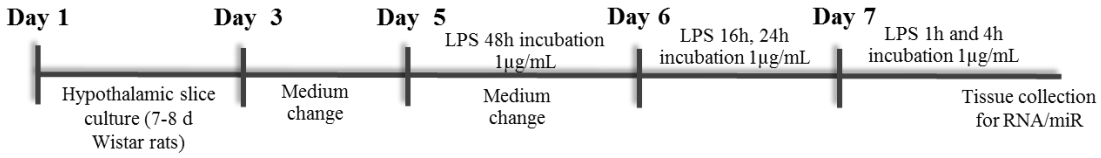

Figure 3.6 – Hypothalamic slice culture and LPS incubation timeline. Hypothalamic slices were cultured for a total of 7 days. Slices were incubated with 1µg/mL of LPS for 1h, 4h, 16h, 24h and 48h. Tissue was collected at day 7.

LPS increases IL1β mRNA in hypothalamic slice cultures

Firstly, to investigate if LPS induced inflammation in hypothalamic slices we measured the mRNA expression levels of pro-inflammatory cytokine IL1β and also IKBKB, which is involved in the inflammatory canonical pathway that leads to the activation of NF-κB [100]. A significant increase of IL1β mRNA levels was observed 1h after LPS incubation, which confirms the activation of this pro-inflammatory pathway (Figure 3.7-**A**). We did not observe any significant differences in the IKBKB mRNA levels in the time-points analysed (Figure 3.7-**B**).

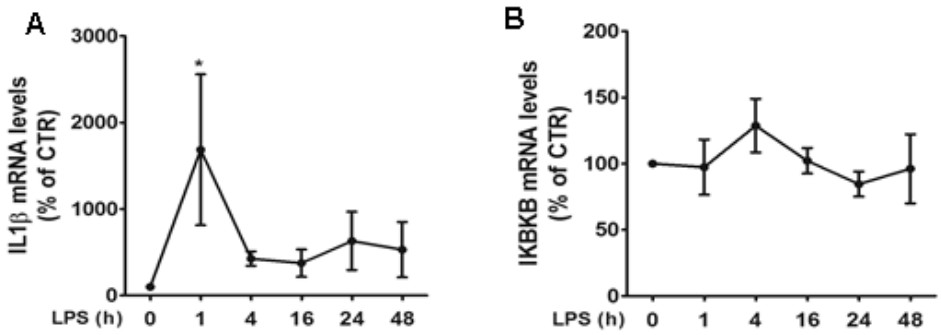

Figure 3.7 – LPS activates pro-inflammatory cytokine in hypothalamic slice cultures. Increased expression of IL1β after 1h incubation with 1µg/mL LPS **(A)** and no differences in IKBKB expression levels **(B)**. n=5, One-Way ANOVA; $p>0.05$; * $p<0.05$, compared to 0h of LPS incubation.

LPS induced inflammation does not alter miRISC genes

Since HFD causes alteration of miRISC genes expression levels in the rat hypothalamus, we investigated if inflammation induced by LPS would also alter their levels in hypothalamic slice cultures. The expression levels of Dgcr8, Ddx6, Ago2, Fmr1 and PabpC1 were evaluated by qRT-PCR, but no differences were observed for the expression levels (Figure 3.8– **A**, **B**, **C**, **D**, and **E**).

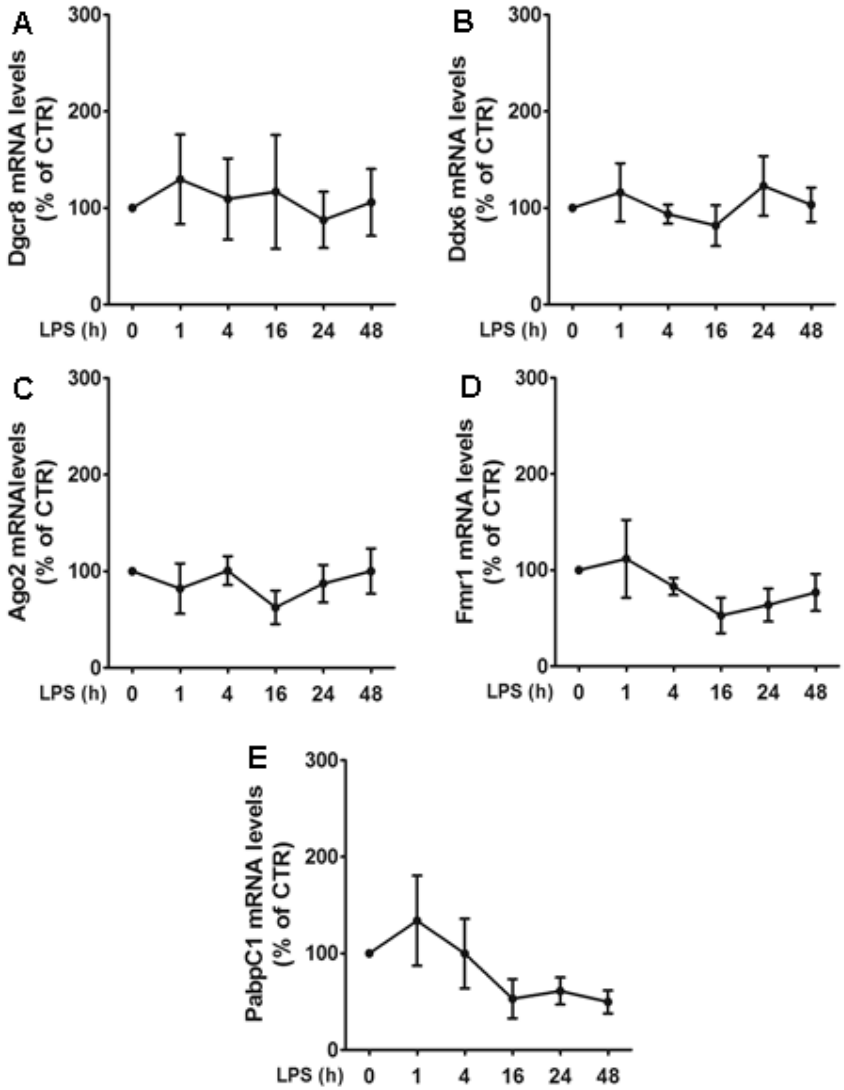

Figure 3.8 – Expression levels of miRISC genes are not altered in hypothalamic slice cultures after LPS exposure. LPS 1µg/mL incubation did not alter mRNA expression levels of Dgcr8 **(A)**, Ddx6 **(B)**, Ago2 **(C)**, Fmr1 **(D)** and PabpC1 **(E)**. n=5, One-Way ANOVA, p>0.05, compared to 0h LPS.

LPS incubation alters expression levels of let-7b and Lin28b

Altered expression levels of let-7b, let-7c and let-7e were detected in the hypothalamus of rats in a HFD regime. Moreover Lin28b mRNA expression levels were also altered in an inverse manner of let-7b alterations. Therefore, we evaluated whether inflammation, via LPS incubation, could trigger equivalent alterations in hypothalamic slices.

With LPS incubation, let-7b expression (Figure 3.9-**A**) increased 4h after the stimulus compared to control levels (0h) and decreased at 24h and 48h in comparison to the peak at 4h. Regarding Lin28b (Figure 3.9-**B**), it was detected a decrease of mRNA levels at 4h, 24h and 48h compared to control levels (0h). Interestingly, the let-7b and Lin28b alterations induced by LPS incubation in hypothalamic slice cultures show a similar pattern to the one observed in the rat hypothalamus upon HFD regime. No changes were observed in let-7c (Figure 3.9-**C**) or let-7e expression levels (Figure 3.9-**D**).

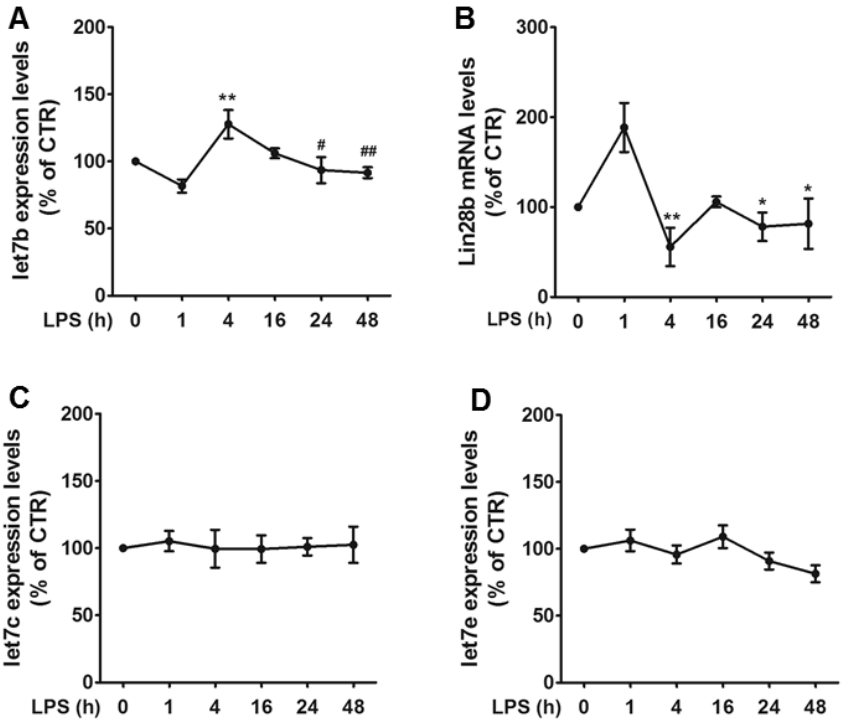

Figure 3.9 – LPS induces alterations in the let-7b/Lin28b system in hypothalamic slice cultures. LPS 1µg/mL altered let-7b expression levels **(A)**, and Lin28b mRNA levels **(B).** Expression levels of let-7c **(C)** and let-7e **(D)** showed no alterations upon 1µg/mL LPS incubation. n=5, One-Way ANOVA, * $p<0.05$; ** $p<0.01$ compared to 0h LPS; #, $p<0.05$; ##, $p<0.01$, compared to 4h LPS.

3.3. Let-7b overexpression in the hypothalamus and physiological implications in obesity

Our data showing the alterations of hypothalamic let-7b and related protein Lin28b upon HFD regime and the equivalent alterations induced by inflammation in hypothalamic slice cultures raised the question to what is the role and effect of let-7b microRNA in HFD induced obesity? Moreover, knowing that let-7b has anti-inflammatory actions [93], it is involved in glucose sensitivity [91] and it promotes neuronal differentiation [70, 89], we hypothesize that let-7b over-expression in the hypothalamus would have a protective effect against HFD induced metabolic and neuropathological alterations.

In order to investigate this, we overexpressed let-7b in the hypothalamus of mice using lentiviral vectors and then submit the mice to an HFD. Of notice, this mice model (lentiviral injection targeting the medial hypothalamus) was used because it was previously established in our laboratory.

For the overexpression of let-7b, the let-7b precursor was cloned into a lentiviral plasmid (Figure 3.10-**A**). For the control, miR-neg with a random sequence, previously validated not to target any mice, rat or human mRNA transcripts [94] was also cloned to a lentiviral plasmid (Figure 3.10-**B**). Both let-7b and miR-neg were expressed under the control of H1 promoter and the plasmid included a reporter gene (GFP) under the control of PGK promoter.

Let-7b plasmid (H1-let-7b) was validated by transfection in HEK239 cell line and quantification of let-7b expression by qRT-PCR (Figure 3.10-**C**). A 1000-fold increase of let-7b levels was achieved in cell transfected with H1-let-7b plasmid when compared with cells transfected with control H1-miR-neg plasmid (Figure 3.10-**D**).

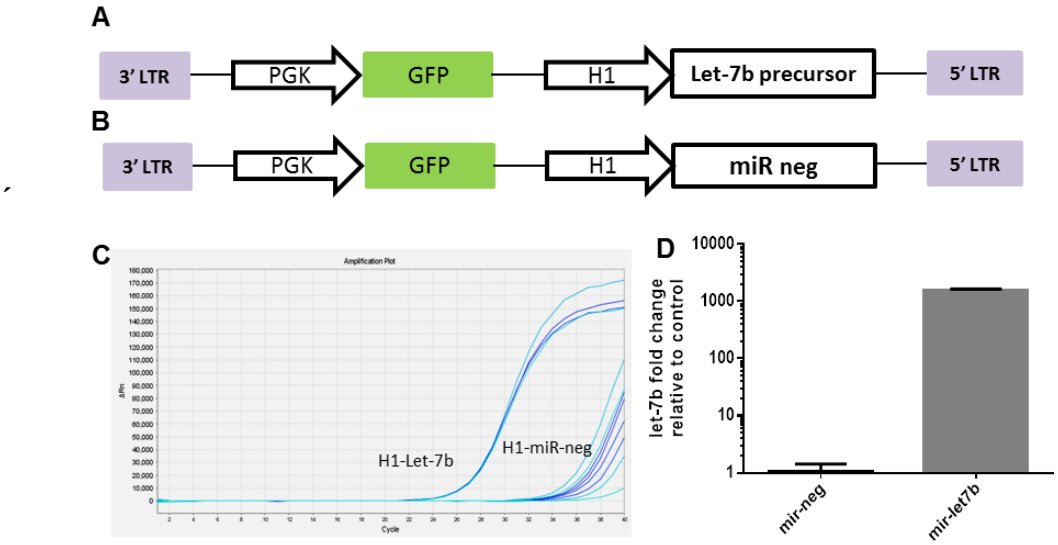

Figure 3.10 – Plasmid constructed for overexpression of let-7b and control miR-neg.
Schematic representation of the plasmids constructed to overexpress microRNA let-7b (H1-let-7b) **(A)**, and the control plasmid constructed to overexpress a random sequence (H1-miR-neg) **(B),** in a lentiviral backbone (3´LTR and 5´LTR), with reporter gene GFP. Amplification plot of qRT-PCR run for let-7b from cells transfected with H1-let-7b or control H1-miR-neg plasmids **(C)**. Quantification of let-7b levels in HEK293 cell line transfected with H1-let-7b or H1-miR-neg **(D)**.

Let-7b overexpression in the hypothalamus

In order to assess the possible anti-obesity effect of let-7b, we overexpress let-7b microRNA in the mouse medial hypothalamus by means of stereotaxic delivery of lentiviral particles (Figure 3.11-**B**). The injection site was evaluated by visualization of reporter gene GFP and it was observed GFP fluorescence bilaterally in the medial hypothalamus (Figure 3.11-**B**).

For this experiment 4 experimental groups were used:

1. **Chow group**; mice in a chow diet (8% fat),
2. **HFD group**, mice in a HFD regime (40% fat),
3. **HFD+let-7b group**, mice with hypothalamic over-expression of let-7b in a HFD regime,

4. **HFD+miR-neg group**, mice with hypothalamic over-expression of miR-neg in a HFD regime

The HFD was initiated one week after the stereotaxic surgery for groups 2, 3 and 4 at the same time, and maintained for 7 weeks (Figure 3.11-**A**). Mice of group 1 were maintained in chow diet for the same period.

Additionally, let-7b overexpression was confirmed by comparing the let-7b expression levels in the hypothalamus of HFD+let-7b mice with HFD+miR-neg mice by qRT-PCR. It was observed a 20% increase in the expression levels of let-7b in mice injected with lentiviral particles to over-express let-7b (Figure 3.11–**C**).

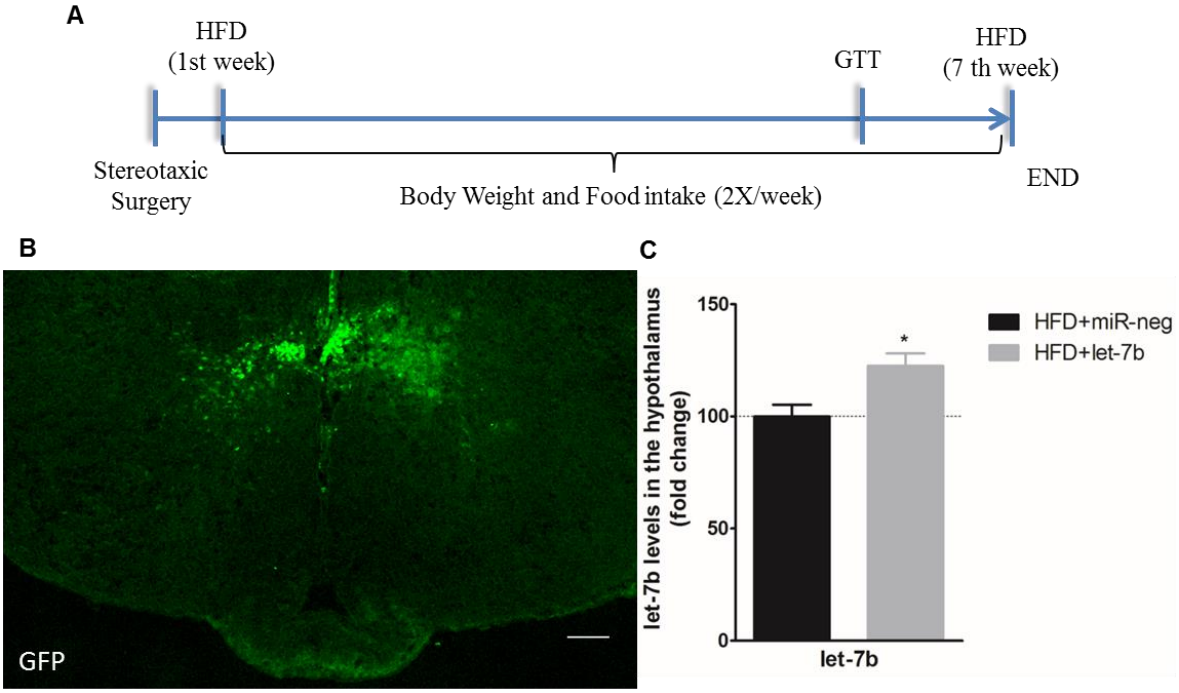

Figure 3.11 – Let-7b overexpression in the mice hypothalamus. Layout of the in vivo experiment **(A)**. Stereotaxic injection of lentiviral particles to over-express let-7b in the mouse hypothalamus visualized by GFP reporter protein. Scale bar, 100µm **(B)**. Quantification of let7-b expression levels in the hypothalamus of mice from HFD+let-7b+ group and HFD+miR-neg group **(C)**. n=3-4, Unpaired T-test, * $p<0.05$, compared to HFD+miR-neg.

Regarding the HFD group, near the experiment end the animals were exhibiting wounds from fighting and decreased body weight gain. Measures were taken to minimize this

behaviour (environment enrichment and animals' separation) but the alterations continued. For this reason, the HFD group was not included in our study analyses.

Consequently, we assumed for the following analysis that the HFD+miR-neg group would be used both as HFD control and microRNA stereotaxic injection control. Thus, the following analyses were performed:

- Comparison between chow group and HFD+miR-neg group, in order to evaluate the alterations induced by HFD
- and, comparison between HFD+let-7b group and HFD+miR-neg group to assess if hypothalamic let-7b overexpression may improve the HFD induced alterations.

Hypothalamic let-7b overexpression ameliorates HFD induced metabolic alterations

Mice on HFD regime (HFD+miR-neg) showed increased cumulative body weight gain when compared to chow animals over the 7 weeks analysed (Figure 3.12-**A**). But when comparing animals in HFD, the group with let-7b overexpression in the hypothalamus showed a slower increase of body weight gain (Figure 3.12-**C**).

Regarding the amount of white adipose tissue (WAT) as percentage of body weight, an increase was observed when comparing chow animals with animals in HFD regime (Figure 3.12-**B**), and no differences between animals in HFD (HFD+miR-neg) and let-7b injected animals in a HFD (Figure 3.12-**D**). Therefore, we can conclude that hypothalamic let-7b overexpression was able to slow down body weight gain in mice in a HFD regime.

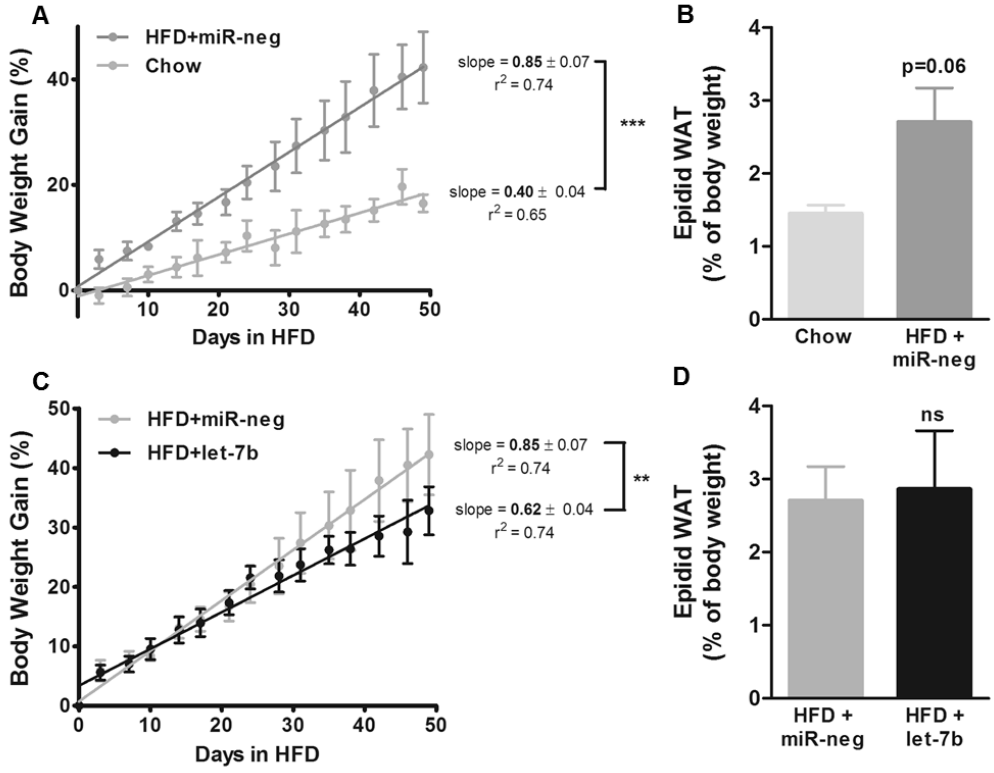

Figure 3.12 – Body weight alterations induced by HFD are ameliorated with hypothalamic let-7b overexpression but WAT accumulation is not changed. Cumulative body weight gain in HFD mice (HFD+miR-neg) compared to chow **(A)** and HFD+miR-neg compared to hypothalamic let-7b overexpression in a HFD regime (HFD+let-7b) **(C)**. Epididymal (Epidid) WAT weight shown as percentage of total body weight of chow and HFD (HFD+miR-neg) mice **(B);** and, HFD+miR-neg and hypothalamic let-7b injected mice in a HFD (HFD+let-7b) **(D)**. n=4-6.For body weight, non-linear regression with F-test; ** $p<0.01$, *** $p<0.001$, compared to chow or HFD+miR-neg. For epididymal WAT, Unpaired T-test, ns $p>0.05$ compared to chow or HFD+miR-neg.

Food intake was measured twice a week for a total of 7 weeks. It was observed an extensive increase of caloric consumption in HFD animals (HFD+miR-neg) compared to chow diet animals during the 7 weeks (Figure 3.13-**A**), resulting in a higher total caloric consumption (Figure 3.13-**B**). However, when comparing mice overexpressing miR-neg in HFD with mice overexpressing let-7b in HFD, it is observed a decrease in the total caloric intake (Figure 3.13-**D**). Surprisingly, daily food intake measured every week was lower in HFD+let-7b group only in weeks 1 and 7 when compared to

HFD+miR-neg animals (Figure 3.13-**C**). At week 3, mice overexpressing let-7b even had increased daily caloric intake. Nevertheless, these data show a decrease in total caloric intake when let-7b is overexpressed in the mouse hypothalamus.

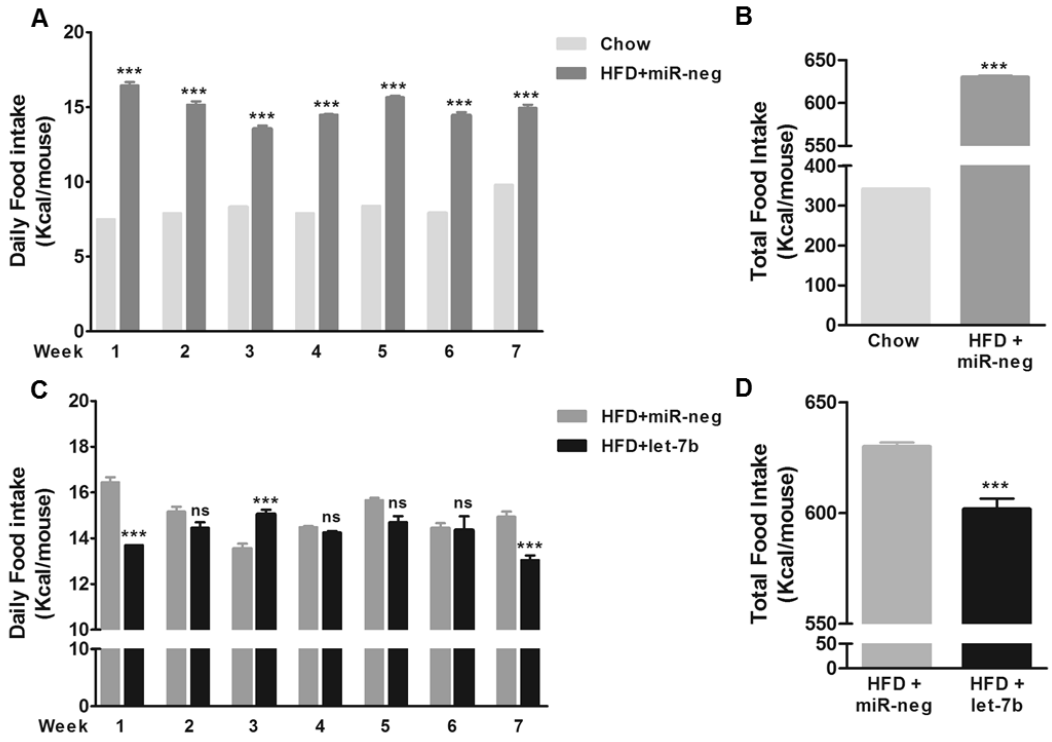

Figure 3.13 – Hypothalamic let-7b overexpression reduces food intake induced by HFD.

Daily food intake (Kcal/mouse) in chow mice and HFD+miR-neg mice for seven weeks **(A)** and total food intake for the 7 weeks analysed **(B)**. Comparison between miR-neg overexpressing mice in a HFD regime and let-7b overexpressing animals also in HFD for daily food intake (Kcal/mouse) **(C)** and total food intake for the 7 weeks analysed **(D)**. n=4-6, Unpaired T-test, ns $p>0.05$, *** $p<0.001$, compared to chow or HFD+miR-neg.

To further characterize the mice metabolic state, glucose tolerance test was performed. This is an indicator of the organism response to glucose delivery and an indication of the diabetic state. We observed that the blood glucose levels curve of HFD animals (HFD+ miR-neg) was higher when compared to chow diet animals (Figure 3.14-**A**), being of significance at 60 minutes after glucose injection. Accordingly, area under the curve (AUC) analyses, in HFD animals showed an increase compared to chow mice

(Figure 3.14-**B**). The HFD animals at the end of the test (120 min) returned to the initial values.

Mice overexpressing let-7b in HFD regime showed improved response to glucose when compared to control mice (HFD+miR-neg) (Figure 3.14-**C**), as can be seen by the significantly lower blood glucose levels at 60 min after glucose injection and a decrease of the area under the curve (Figure 3.14-**D**). Therefore, we can conclude that hypothalamic let-7b overexpression contributes to the improvement of glucose clearance in obese mice.

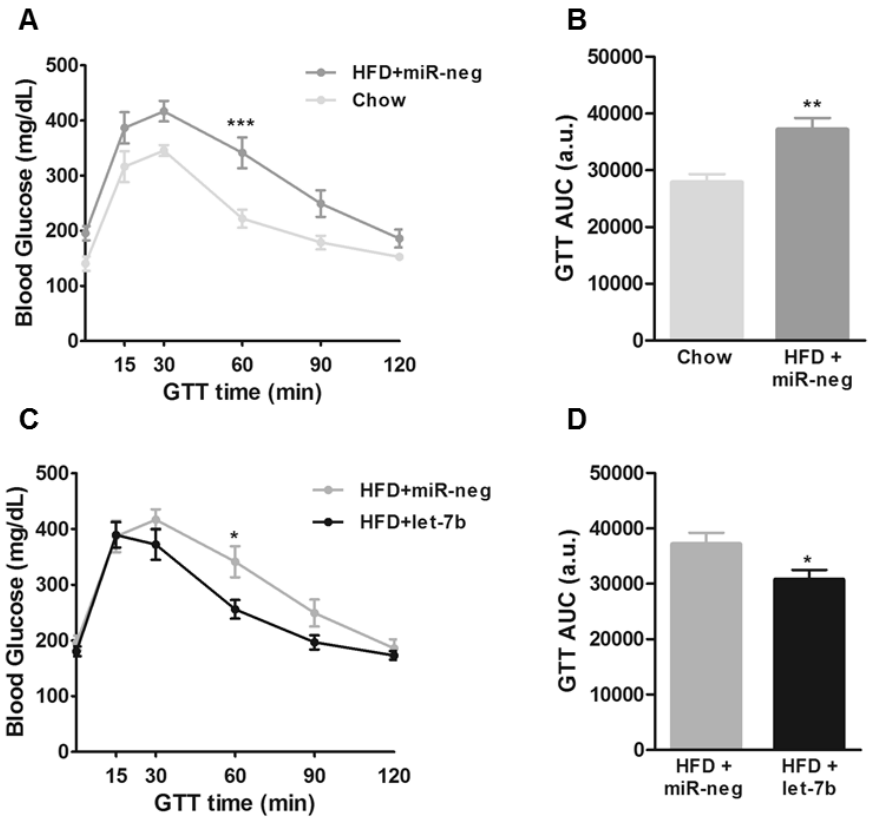

Figure 3.14 – Glucose response alteration induced by HFD is ameliorated with hypothalamic let-7b overexpression. Blood glucose levels in HFD mice (HFD+miR-neg) compared to chow during the GTT test **(A),** and area under the curve **(B).** Blood glucose levels in mice overexpressing miR-neg in HFD (HFD+miR-neg) compared to mice overexpressing let-7b in a HFD (HFD+let-7b) during the GTT test **(C)** and area under the curve **(D)**. n=4-6; GTT curve, two-way ANOVA, * $p<0.05$, *** $p<0.001$, compared to chow or HFD+miR-neg; AUC, Unpaired T-test, * $p<0.05$, ** $p<0.01$, compared to chow or HFD+miR-neg.

HFD and hypothalamic let-7b overexpression do not cause alterations of microRNA pathway in the hypothalamus

Similarly to the experiments conducted in rats, the expression levels of miRISC genes were investigated. HFD did not alter miRISC genes, Dicer, Drosha and Exportin5 in the mice hypothalamus (Figure 3.15-**A**). Similarly, hypothalamic let-7b overexpression did not change the expression levels of these genes (Figure 3.15-**B**).

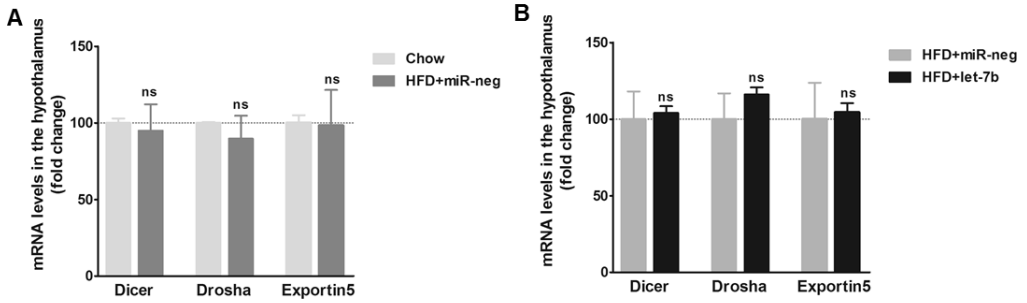

Figure 3.15 –miRISC genes expression in the hypothalamus are not altered by HFD or let-7b overexpression. miRISC genes expression levels in chow and HFD (HFD+miR-neg) mice **(A)** and, miR-neg and let-7b overexpressing mice in HFD regime **(B)**. n=3/4, Unpaired T-test ns p>0.05, compared to chow or miR-neg_HFD.

Let-7 microRNAS (let-7b, let-7c and let-7e) expression levels were also investigated and it was observed that HFD regime did not alter their expression in the mice hypothalamus (Figure 3.16-**A**). Also, hypothalamic let-7b overexpression did not alter the levels of let-7c and let-7e (Figure 3.16-**B**). In this group (HFD+let-7b) there was a 20% increase in hypothalamic let-7b levels due to lentiviral delivery of let-7b precursor, as described above.

Finally, the mRNA levels of Lin28b were also evaluated for it is an important regulator of let-7 biogenesis. HFD regime (HFD+miR-neg) compared to chow alters Lin28b expression levels (Figure 3.16-**C**). However when let-7b was overexpressed in the hypothalamus, no alterations were detected (Figure 3.16-**D**).

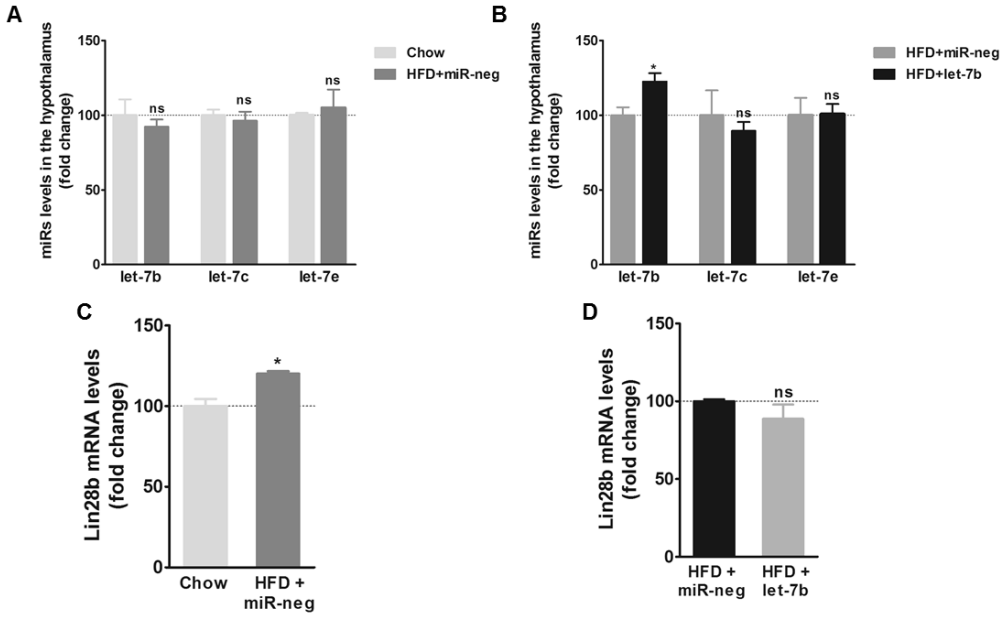

Figure 3.16 – Lin28b expression is altered with HFD and let-7 microRNAs expression in the hypothalamus is not altered by HFD or let-7b overexpression. Expression levels of let-7 microRNAs **(A)** and Lin28b **(B)** in mice fed with chow or HFD (HFD+miR-neg). Expression levels of let-7 microRNAs **(C)** and Lin28b **(D)** in mice fed with miR-neg overexpression (HFD+miR-neg) or let-7b overexpression in HFD. n=3-4; Unpaired T-test, ns $p>0.05$, * $p<0.05$, compared to chow or HFD+miR-neg.

Hypothalamic let-7b overexpression decreases pro-inflammatory markers activated in HFD

High caloric diet instigates the activation of pro-inflammatory pathways in rodent hypothalamus [8, 9]. Moreover, we have previously observed changes in let-7b expression in inflammatory conditions in hypothalamic slice cultures (Figure 3.9-**C**). Therefore, in this study, we evaluated whether let-7b could prevent the activation of such hypothalamic inflammatory pathways in mice under an HFD regime.

HFD leads to increase levels of inflammatory cytokines IL1β and IL6 in the mice hypothalamus as shown by increased mRNA expression levels (Figure 3.17-**A**). Interestingly, overexpression of let-7b in the mice hypothalamus with a HFD led to a decrease of IL1β and IL6 expression and of gliosis marker, GFAP (Figure 3.17-**B**), showing a reduction in inflammation.

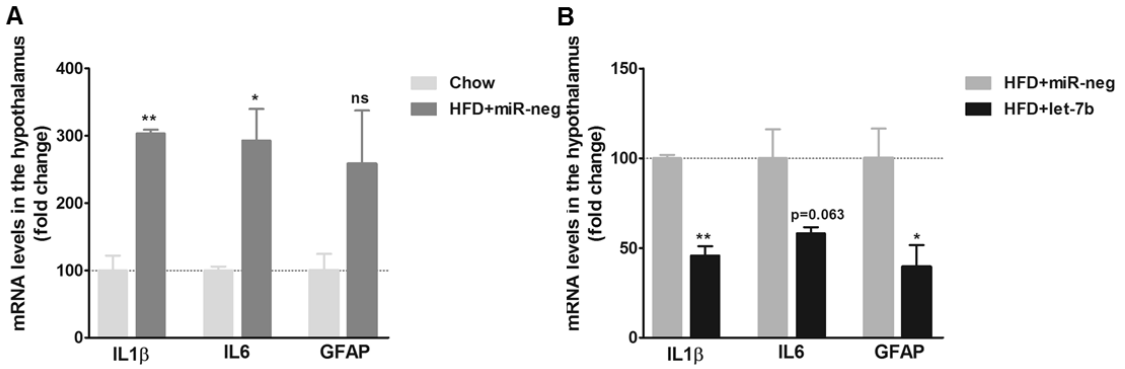

Figure 3.17- Hypothalamic let-7b overexpression decreases inflammatory markers increase induced by HFD in mice hypothalamus. IL1β, IL6 and GFAP expression levels in the mice hypothalamus in HFD **(A)** and with let-7b overexpression **(B)**. n=3-4; Unpaired T-test, ns $p>0.05$, * $p<0.05$, ** $p<0.01$, compared to chow or HFD+miR-neg.

Hypothalamic let-7b overexpression decreases POMC levels in mice in HFD regime

Neuropeptides in the hypothalamus regulate feeding behaviour being of interest their expression levels in animals in HFD. Compared to chow diet, HFD (miR-neg+HFD) did not alter NPY, AgRP or POMC levels in the mice hypothalamus (Figure 3.18-**A**). However, with let-7b overexpression it was observed a decrease of anorexigenic neuropeptide POMC mRNA expression levels in the hypothalamus (Figure 3.18-**B**). The orexigenic neuropeptides evaluated (NPY and AGRP) were not altered in HFD+let-7b group (Figure 3.18-**B**).

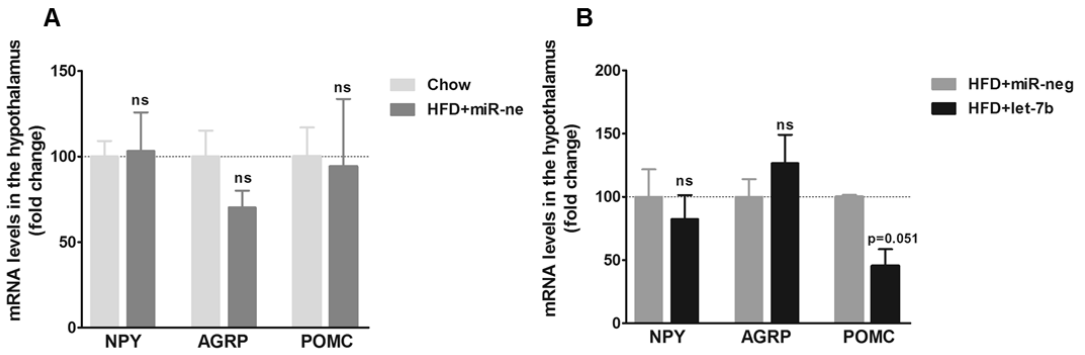

Figure 3.18 – HFD does not alter NPY, AgRP or POMC mRNA levels in the mice hypothalamus but let-7b overexpression decreases POMC mRNA levels. The mRNA levels of NPY, AgRP and POMC in the hypothalamus of mice under HFD **(A)** and HFD upon let-7b overexpression **(B)**. n=3-4; Unpaired T-test, ns $p>0.05$, compared to chow or HFD+miR-neg.

Hypothalamic let-7b overexpression decreases IRS1 levels in HFD regime

HFD leads to hypothalamic insulin and leptin resistance [10]. Moreover, it is known that let-7 microRNAs are involved in the regulation of insulin pathway components, and have been associated with the development of insulin resistance [70].

Therefore, we analysed the expression of insulin receptor (InsR) and insulin receptor substrate 1 (IRS1) to assess modifications in insulin pathway activity, and leptin receptor (LepR) for alterations in leptin activity. When comparing chow mice with HFD (HFD+miR-neg) we did not observe significant mRNA expression alterations (Figure 3.19-**A**). However, with let-7b overexpression there is a decrease of IRS1 mRNA expression levels, but no significant changes of InsR or LepR (Figure 3.19-**B**).

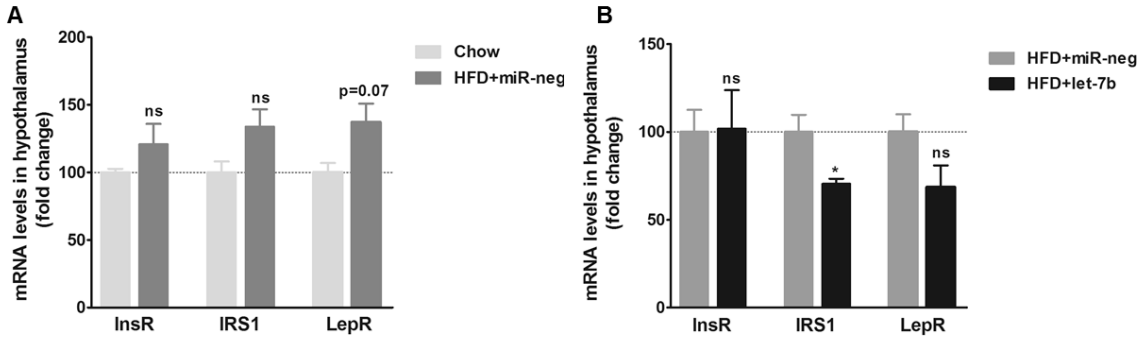

Figure 3.19 – HFD does not alter InsR, IRS1 or LepR mRNA levels in the mice hypothalamus but let-7b overexpression decreases IRS1 levels. Expression levels of InsR, IRS1 and LepR in the hypothalamus of HFD **(A)** and HFD upon let-7b overexpression **(B)**. n=3-4; Unpaired T-test, ns $p>0.05$, * $p<0.05$, compared to chow or HFD+miR-neg.

Hypothalamic let-7b overexpression prevents the increase of cell proliferation markers in the hypothalamus induced by HFD

In previous experiments we observed alterations of neurogenic activity in the hypothalamus of rats upon HFD (see Figure 3.4). Moreover, let-7b is known to be involved in neuronal development, promoting differentiation [83]. Therefore, we evaluated neurogenic activity in the hypothalamus of mice in HFD regime and upon hypothalamic let-7b overexpression. For this we evaluated the mRNA expression levels of proliferative marker (PCNA), neuroprogenitor cell markers (Notch1, Mash1 Musashi1), neurogenic markers (BDNF, IGF-1 and NeuroD1). In mice in a HFD regime (miR-neg+HFD), Notch1 and Musashi1 (MS1) are increased, but no alterations were observed in Mash1, PCNA, IGF-1, BDNF or Neurogenic Differentiation1 (NeuroD1) mRNA levels in the mice hypothalamus (Figure 3.20-**A**). In contrast, hypothalamic overexpression of let-7b led to a decrease of Notch1 mRNA levels, but with no expression changes in Mash1, MS1, PCNA, IGF-1, BDNF or ND1 mRNA levels were observed (Figure 3.20-**B**).

These data suggest that hypothalamic let-7b overexpression can partially contrary the alterations in neurogenic activity induced by HFD regime in mice.

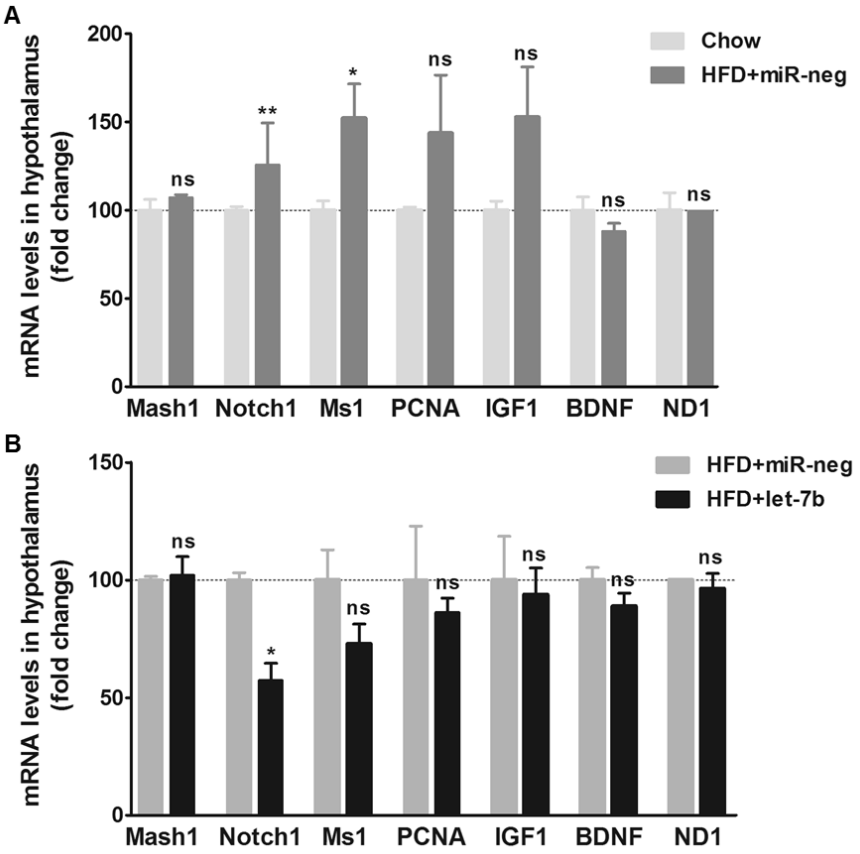

Figure 3.20 – HFD increases neuroprogenitor cells marker Notch1 and MS1 that hypothalamic let-7b overexpression prevents. Neurogenic markers mRNA expression levels in the hypothalamus of mice in HFD regime **(A)** and with let-7b overexpression **(B)**. n=3-4; Unpaired T-test, ns $p>0.05$, * $p<0.05$, ** $p<0.01$, compared to chow or HFD+miR-neg.

Chapter IV. Discussion

Obesity and overweight are major public health matters with a big impact in our society. Obesity is characterized by a body mass index (kg/m^2), of 30 or more, which implies an increase of about 25% body weight gain. Additionally increased body weight results from excessive fat accumulation and it can be accompanied by the development of high blood pressure, high cholesterol and insulin resistance [1].

In obesity studies, the most commonly used animal model is the diet induced obesity because it mimics the most prevalent cause of obesity in humans. In these models, animals are placed on a high fat diet regime, with fat content ranging from 20% to 60%, for different periods of time. The body weight gain is gradual, and is noticeable after short periods (two weeks) [101]. Furthermore in these obesity models are observed hyperphagya, hyperglycemia and hyperinsulinemia [101].

In our study we used two rodent species, Wistar rats and C57BL/6 mice, both obesity prone when in HFD regime [101]. Both the species showed increased body weight higher that 25% after 7-8 weeks of 40% fat diet regime, being considered as obese. Additionally the mice under HFD were hyperglycemic and had increased fat accumulation; this specific strain is very susceptible to insulin resistance and obesity for its isogenic characteristics. All in all both our animal models developed obesity induced by high fat diet consumption.

MicroRNAs are regulatory elements important in peripheral metabolism regulation but their putative role in hypothalamic physiology and obesity has not been elucidated yet.

Our analysis shows for the first time impairment in hypothalamic miRNA function after HFD regime. In fact, in obese rats there is alteration of miRNA biogenesis genes, at the two time points analysed, short period (4 weeks HFD) and long period (8 weeks HFD). Previous studies have indicated that energetic state influences miRNA biogenesis in the hypothalamus [74, 77, 79]. In anorexia animal model mice was observed up-regulation

of miRISC genes [74] Dicer alterations (reduction of expression levels) are associated with obesity and hyperphagya [77, 79].

Moreover, the miRISC alterations we observed were not limited to the hypothalamus, since alterations were observed in the cortex as well. Previous studies in our laboratory indicated alterations in the expression levels of specific microRNAs in the hypothalamus of obese rats, particularly the let-7 microRNA family. Further analysis conducted during this project revealed that let-7b expression decreased in short term HFD followed by an increase in longer periods. Moreover Lin28b, a target protein of let-7 microRNAs, presented the opposite expression pattern. Other let-7 family miRs, let-7c and let-7e were altered in obesity but did not present this pattern. In the cortex however the alterations of let-7 miRNAs and Lin28b are only observed after long term HFD and to a minor extent that in the hypothalamus.

The inverse expression pattern of Lin28b and let-7b in the rat hypothalamus depending of high fat diet period is of particular interest because of the key regulatory roles of this system, such as neuronal development, metabolic functions and inflammation [83].

Regarding neuronal features, neurogenesis is a highly regulated, transient process that is altered by energy availability [20, 37, 46, 48]. Hypothalamic neurogenesis in particular, has been appointed has a protective mechanism activated in the first days of HFD to respond to metabolic challenges, that in a continued consumption results in reduction of progenitor cells proliferative capacity [9].

After short HFD period (4 weeks), Musashi1 and SOX2 protein levels were increased in the rat hypothalamus, suggesting neurogenesis activation. However in longer periods protein levels returned to control (chow) levels. Moreover, no alterations were observed in proliferative marker PCNA perhaps because cell proliferation occurred previously to the short period (4 weeks) analysed. Our data are in accordance to previous studies

showing neurogenesis activation in the hypothalamus as early response to fat consumption [8].

In the cortex, no changes were observed in the neurogenic markers evaluated, possibly because it is not responsive to diet alterations.

Previous studies showed that hypothalamic neurogenic activity alteration is due to inflammation activation occurring after HFD consumption [30]. For example IKKβ and NF-κB through activation of Notch1 prevents differentiation causing neurons to present lower neurogenic capacity [30]. Furthermore, activation of inflammatory factors occurs in the first days of HFD but is then reduced and maintained at low levels for 4 weeks [9], later on, the levels of inflammatory factors in the rodent hypothalamus increased once again [9]. This evidence may explain our observations such that after 4 weeks of HFD, a reduction of inflammation may allow neurogenesis activation but, after 8 weeks of HFD, inflammation is settled and thus preventing neurogenesis increase required for response to diet challenge.

In high fat diet, fatty acids cause inflammation, inducing endoplasmic reticulum stress, mitochondrial dysfunction and ultimately apoptosis, leading to neuronal loss [8, 9]. Let-7b/Lin28b system interacts with pro-inflammatory factors, regulating interleukins directly or through NF-κB regulation [93].

Indeed, let-7b and Lin28b present a similar expression pattern in an inflammatory state as in HFD conditions as observed when LPS was used as an inflammation instigator in hypothalamic slice cultures. Besides, inflammatory pathway activated by LPS [99] and fatty acids present in HFD [27] are the same, there is activation of TLR4 that initiates NF-κB and IKKβ pathways.

Taking into account our analysis, along with let-7b role in metabolism, inflammation and neuronal development [83] led us to hypothesize that let-7b miRNA may have a protective effect in obesity.

With lentiviral vectors technology deployed in our laboratory, let-7b was overexpressed in the hypothalamus of mice that were afterwards subjected to HFD. We successfully achieved a 20% overexpression let-7b in the medial hypothalamus. The expression levels of other let-7 microRNAs evaluated (let-7c and let-7e) in the mouse hypothalamus were not altered by HFD consumption or let-7b overexpression. Moreover, when comparing chow mice with HFD mice we did not observe changes in let-7b expression but Lin28b was however increased in HFD mice (7 weeks HFD). A decrease of Lin28b levels due to let-7b overexpression was expected [57] but, surprisingly, the mRNA levels were not changed in mice with let-7b overexpression. Nevertheless, it is possible that the expected decrease is only observed at the protein level.

In addition, miRISC genes were not differentially expressed in the hypothalamus of mice in HFD regime or when let-7b was overexpressed. Together, these evidence show that the alterations induced by HFD in the mouse hypothalamus are not similar to the ones observed in rat in what concerns the microRNA pathway (miRISC genes and let-7b/Lin28b system). We hypothesize that alterations present in rats were not detected in the time-points of analyzes for mice and, also, the genes analysed regarding miRISC were not the same. In this study let-7b isoform was overexpressed in the hypothalamus of mice in a HFD regime for a long term period (7 weeks) to evaluate the possible protective effect in what concerns metabolic and neuropathological alterations caused by obesity.

Concerning body weight gain, we observed a cumulative higher increase with HFD consumption in HFD+miR-neg mice. However, in let-7b overexpressing mice in the same HFD regime, the increase was slower and to a minor extent. Caloric intake, on the overall, was also reduced in let-7b mice. Concerning WAT accumulation, comparing chow animals and HFD we observe a higher accumulation consistent with obesity. The overexpression of let-7b did not alter this increase despite the body weight and caloric intake being lower. However is stated that in obesity apart from an increase of WAT accumulation, the tissue is not healthy and presents hypoxia, impaired angiogenesis and inflammation [5]. If the accumulation, however is adequate it maintains the healthy state of the tissue, specifically suitable expansion, angiogenesis and tissue remodeling preventing the development of other diseases [5]. Thus, further analyses to the WAT of these mice are required to assess the effect of let-7b overexpression in the health state of WAT.

A consequence of obesity is diabetes and impaired glucose clearance leading to high glucose in bloodstream and insulin resistance [3]. Glucose tolerance test (GTT) showed that mice in HFD (both miR-neg and let-7b overexpressing mice) are hyperglycemic (basal levels). However, after glucose delivery, let-7b overexpressing animals showed an improved response compared to HFD animals. Let-7 mice despite being hyperglycemic are capable of clearing glucose more rapidly and effectively than HFD mice.

With these date we questioned whether the improvement in glucose clearance was due to improved peripheral or central glucose metabolism. In fact, the slower increase of body weight and decreased caloric intake of HFD+let-7b mice, compared to miR-neg+HFD mice, could be responsible for better peripheral sensitivity. To investigate this

hypothesis further studies should be performed to assess glucose pathway in glucose target organs (liver, muscle, WAT).

Nevertheless, analyses of mRNA expression levels of insulin and leptin signaling pathway intermediaries, InsR and IRS1 and LepR in the mouse hypothalamus showed no alterations in HFD mice and a reduction of mRNA levels of IRS1 when let-7b was overexpressed.

This decrease however does not contradict the metabolic improvement we observed since insulin signaling pathway is not just IRS1 dependent, other pathways can be activated. Additionally we should assess the expression levels of IRS2, to see if there is a compensation for the decrease of IRS1.

Inflammation is the key mechanism responsible for hypothalamic injury in obesity [8, 9, 22-24, 27-29] but let-7 miRs have already been appointed as anti-inflammatory factors [93]. In accordance, HFD increased the expression levels of interleukins (IL1β and IL6) and gliosis factor GFAP in the mouse hypothalamus. And, importantly, hypothalamic let-7b overexpression decreased IL1β, IL6 and GFAP mRNA expression levels in mice in HFD regime.

Reduction of inflammatory factors with let-7b overexpression supports our hypothesis of let-7b as a putative factor of anti-obesity. Furthermore, it supports the amelioration of glucose response, since inflammatory activation can lead to hypothalamic insulin resistance by activation of TNF-α and IKKβ pathways [23, 28].

Though no differences were observed in neuropeptide mRNAs expression in HFD mice, when let-7b was overexpressed in mice in this regime, there is loss of anorexigenic POMC mRNA levels. This is an intriguing finding when considering the decrease a food intake and body weight gain shown by HFD+let-7b mice. Nevertheless, informatics' tools (Mirna.org and TargetScan.org) have predicted POMC mRNA

transcript has a let-7b target. However to support this hypothesis, luciferase assay should be performed for validation.

Regarding neurogenesis, there was an increase of Notch1 and Musashi1 mRNA levels in HFD compared to chow; that, in contrast, were decreased when let-7b was overexpressed. Since let-7b is a differentiation inducer, the reduction of Notch1, which maintains neuronal cells in proliferating state, was to be expected. Neurogenic markers, Mash1, BDNF, IGF-1 and NeuroD1, as well as proliferative marker, PCNA were not altered by HFD alone or when let-7b was overexpressed. It is important to mention that neurogenesis occurs in the hypothalamus at slow rates and, therefore, it may be technically difficult that assess differences in neurogenesis activity [47]. For this reason, in this study it is crucial to increase the number of samples when assessing neurogenesis markers particularly (but also for other mechanisms evaluated).

To understand the impact of let-7b in hypothalamic neurogenesis and neuronal loss, immunohistochemistry studies should also be conducted in order to identify neurogenic markers positive cells number, hypothalamic neurons number. In-situ hybridization studies could also elucidate in which neural population and cell types let-7b is expressed.

Chapter V. Conclusions

Despite the pointed limitations, we can conclude that HFD regime leads to a deregulation of the hypothalamic microRNA pathway in the rodent hypothalamus and leads to alterations of the let-7/Lin28 system. The involvement of this system in several functions, neurogenesis, inflammation and metabolism makes it an interesting candidate to study in obesity models. Particularly, let-7b and Lin28b since they presented a differential expression pattern in the hypothalamus of HFD rodents dependent of HFD period. Regarding the alterations of let-7/Lin28b system inflammation is responsible for the alterations observed in the hypothalamus of rodents in HFD regime. Moreover, the overexpression of let-7b in the hypothalamus of mice in HFD regime prevented metabolic alterations and hypothalamic neuropathology caused by HFD regime.

The results in the present study suggest that let-7b microRNA could be a new therapeutic target for obesity.

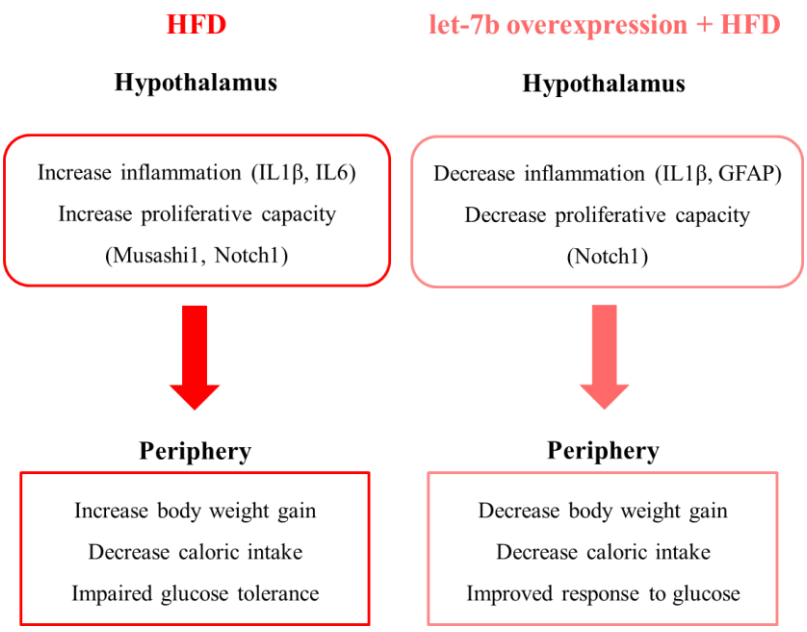

Figure 5.1 – Schematic representation of hypothalamic and periphery response to HFD and let-7 overexpression in the hypothalamus in mice in HFD regime.

Chapter VI. Bibliography

1. Juni, M.H., *OBESITY: A Public Health Threats in Developing Countries.* International Journal of Public Health and Clinical Sciences, 2015. **2**(2).
2. Wellen, K.E. and G.S. Hotamisligil, *Obesity-induced inflammatory changes in adipose tissue.* Journal of Clinical Investigation, 2003. **112**(12): p. 1785.
3. Qatanani, M. and M.A. Lazar, *Mechanisms of obesity-associated insulin resistance: many choices on the menu.* Genes & development, 2007. **21**(12): p. 1443-1455.
4. Cummings, D.E. and M.W. Schwartz, *Genetics and pathophysiology of human obesity.* Annual review of Medicine, 2003. **54**(1): p. 453-471.
5. Sun, K., C.M. Kusminski, and P.E. Scherer, *Adipose tissue remodeling and obesity.* The Journal of clinical investigation, 2011. **121**(6): p. 2094.
6. Teng, K.-T., et al., *Modulation of obesity-induced inflammation by dietary fats: mechanisms and clinical evidence.* Nutrition journal, 2014. **13**(1): p. 12.
7. Cai, D., *Neuroinflammation and neurodegeneration in overnutrition-induced diseases.* Trends in Endocrinology & Metabolism, 2013. **24**(1): p. 40-47.
8. Moraes, J.C., et al., *High-fat diet induces apoptosis of hypothalamic neurons.* PloS one, 2009. **4**(4): p. e5045.
9. Thaler, J.P., et al., *Obesity is associated with hypothalamic injury in rodents and humans.* The Journal of clinical investigation, 2012. **122**(1): p. 153.
10. Yu, J.H. and M.-S. Kim, *Molecular mechanisms of appetite regulation.* Diabetes & metabolism journal, 2012. **36**(6): p. 391-398.
11. Lee, D.A. and S. Blackshaw, *Functional implications of hypothalamic neurogenesis in the adult mammalian brain.* International Journal of Developmental Neuroscience, 2012. **30**(8): p. 615-621.
12. Bouret, S.G., S.J. Draper, and R.B. Simerly, *Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice.* The Journal of neuroscience, 2004. **24**(11): p. 2797-2805.
13. Nilsson, I., et al., *Maturation of the hypothalamic arcuate agouti-related protein system during postnatal development in the mouse.* Developmental brain research, 2005. **155**(2): p. 147-154.
14. Mullier, A., et al., *Differential distribution of tight junction proteins suggests a role for tanycytes in blood-hypothalamus barrier regulation in the adult mouse brain.* Journal of Comparative Neurology, 2010. **518**(7): p. 943-962.
15. Hillebrand, J., D. De Wied, and R. Adan, *Neuropeptides, food intake and body weight regulation: a hypothalamic focus.* Peptides, 2002. **23**(12): p. 2283-2306.
16. Grove, K.L. and M.S. Smith, *Ontogeny of the hypothalamic neuropeptide Y system.* Physiology & behavior, 2003. **79**(1): p. 47-63.
17. Grayson, B., et al., *Critical determinants of hypothalamic appetitive neuropeptide development and expression: species considerations.* Frontiers in neuroendocrinology, 2010. **31**(1): p. 16-31.
18. Stanley, S., et al., *Hormonal regulation of food intake.* Physiological reviews, 2005. **85**(4): p. 1131-1158.
19. Cordeira, J. and M. Rios, *Weighing in the role of BDNF in the central control of eating behavior.* Molecular neurobiology, 2011. **44**(3): p. 441-448.
20. Breton, C., *The hypothalamus–adipose axis is a key target of developmental programming by maternal nutritional manipulation.* Journal of Endocrinology, 2013. **216**(2): p. R19-R31.
21. Cummings, D.E., et al., *A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans.* Diabetes, 2001. **50**(8): p. 1714-1719.
22. Belgardt, B.F. and J.C. Brüning, *CNS leptin and insulin action in the control of energy homeostasis.* Annals of the New York Academy of Sciences, 2010. **1212**(1): p. 97-113.
23. Posey, K.A., et al., *Hypothalamic proinflammatory lipid accumulation, inflammation, and insulin resistance in rats fed a high-fat diet.* American Journal of Physiology-Endocrinology and Metabolism, 2009. **296**(5): p. E1003-E1012.

24. De Souza, C.T., et al., *Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus.* Endocrinology, 2005. **146**(10): p. 4192-4199.
25. El-Haschimi, K., et al., *Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity.* Journal of Clinical Investigation, 2000. **105**(12): p. 1827.
26. Münzberg, H., J.S. Flier, and C. Bjørbæk, *Region-specific leptin resistance within the hypothalamus of diet-induced obese mice.* Endocrinology, 2004. **145**(11): p. 4880-4889.
27. Milanski, M., et al., *Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity.* The Journal of Neuroscience, 2009. **29**(2): p. 359-370.
28. Zhang, X., et al., *Hypothalamic IKKβ/NF-κB and ER stress link overnutrition to energy imbalance and obesity.* Cell, 2008. **135**(1): p. 61-73.
29. Meng, Q. and D. Cai, *Defective hypothalamic autophagy directs the central pathogenesis of obesity via the IκB kinase β (IKKβ)/NF-κB pathway.* Journal of Biological Chemistry, 2011. **286**(37): p. 32324-32332.
30. Li, J., Y. Tang, and D. Cai, *IKKβ/NF-κB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes.* Nature cell biology, 2012. **14**(10): p. 999-1012.
31. Lee, E.B. and R.S. Ahima, *Alteration of hypothalamic cellular dynamics in obesity.* The Journal of clinical investigation, 2012. **122**(1): p. 22-25.
32. Ahmed, S., et al., *Transcription factors and neural stem cell self-renewal, growth and differentiation.* Cell adhesion & migration, 2009. **3**(4): p. 412-424.
33. Zhang, J. and J. Jiao, *Molecular Biomarkers for Embryonic and Adult Neural Stem Cell and Neurogenesis.* BioMed Research International, 2014.
34. Cheng, M.-F., *Hypothalamic neurogenesis in the adult brain.* Frontiers in neuroendocrinology, 2013. **34**(3): p. 167-178.
35. Bolborea, M. and N. Dale, *Hypothalamic tanycytes: potential roles in the control of feeding and energy balance.* Trends in neurosciences, 2013. **36**(2): p. 91-100.
36. Dietrich, M.O. and T.L. Horvath, *Fat incites tanycytes to neurogenesis.* Nature neuroscience, 2012. **15**(5): p. 651-653.
37. Lee, D.A., et al., *Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche.* Nature neuroscience, 2012. **15**(5): p. 700-702.
38. Xu, Y., et al., *Neurogenesis in the ependymal layer of the adult rat 3rd ventricle.* Experimental neurology, 2005. **192**(2): p. 251-264.
39. Pérez-Martín, M., et al., *IGF-I stimulates neurogenesis in the hypothalamus of adult rats.* European Journal of Neuroscience, 2010. **31**(9): p. 1533-1548.
40. Lugert, S., et al., *Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging.* Cell stem cell, 2010. **6**(5): p. 445-456.
41. Arnold, K., et al., *Sox2+ adult stem and progenitor cells are important for tissue regeneration and survival of mice.* Cell stem cell, 2011. **9**(4): p. 317-329.
42. MacNicol, A.M., A. Wilczynska, and M.C. MacNicol, *Function and regulation of the mammalian Musashi mRNA translational regulator.* Biochemical Society Transactions, 2008. **36**(Pt 3): p. 528.
43. Pencea, V., et al., *Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus.* The Journal of Neuroscience, 2001. **21**(17): p. 6706-6717.
44. Daftary, S.S. and A.C. Gore, *IGF-1 in the brain as a regulator of reproductive neuroendocrine function.* Experimental Biology and Medicine, 2005. **230**(5): p. 292-306.
45. Severi, I., et al., *Opposite effects of a high-fat diet and calorie restriction on ciliary neurotrophic factor signaling in the mouse hypothalamus.* Frontiers in neuroscience, 2013. **7**.
46. Kokoeva, M.V., H. Yin, and J.S. Flier, *Neurogenesis in the hypothalamus of adult mice: potential role in energy balance.* Science, 2005. **310**(5748): p. 679-683.

47. Sousa-Ferreira, L., L.P. de Almeida, and C. Cavadas, *Role of hypothalamic neurogenesis in feeding regulation.* Trends in Endocrinology & Metabolism, 2014. **25**(2): p. 80-88.
48. McNay, D.E., et al., *Remodeling of the arcuate nucleus energy-balance circuit is inhibited in obese mice.* The Journal of clinical investigation, 2012. **122**(1): p. 142.
49. Pierce, A.A. and A.W. Xu, *De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance.* The Journal of Neuroscience, 2010. **30**(2): p. 723-730.
50. Asuelime, G.E. and Y. Shi, *The little molecules that could: a story about microRNAs in neural stem cells and neurogenesis.* Frontiers in neuroscience, 2012. **6**.
51. Xie, H., L. Sun, and H.F. Lodish, *Targeting microRNAs in obesity.* 2009.
52. Carthew, R.W. and E.J. Sontheimer, *Origins and mechanisms of miRNAs and siRNAs.* Cell, 2009. **136**(4): p. 642-655.
53. Laterza, O.F., et al., *Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury.* Clinical chemistry, 2009. **55**(11): p. 1977-1983.
54. Weiland, M., et al., *Small RNAs have a large impact: circulating microRNAs as biomarkers for human diseases.* RNA biology, 2012. **9**(6): p. 850-859.
55. Meister, B., S. Herzer, and A. Silahtaroglu, *MicroRNAs in the Hypothalamus.* Neuroendocrinology, 2013. **98**(4): p. 243-253.
56. Davis-Dusenbery, B.N. and A. Hata, *Mechanisms of control of microRNA biogenesis.* Journal of biochemistry, 2010. **148**(4): p. 381-392.
57. Roush, S. and F.J. Slack, *The let-7 family of microRNAs.* Trends in cell biology, 2008. **18**(10): p. 505-516.
58. Trajkovski, M. and H. Lodish, *MicroRNA networks regulate development of brown adipocytes.* Trends in Endocrinology & Metabolism, 2013. **24**(9): p. 442-450.
59. Heneghan, H., et al., *Differential miRNA expression in omental adipose tissue and in the circulation of obese patients identifies novel metabolic biomarkers.* The Journal of Clinical Endocrinology & Metabolism, 2011. **96**(5): p. E846-E850.
60. Krützfeldt, J. and M. Stoffel, *MicroRNAs: a new class of regulatory genes affecting metabolism.* Cell metabolism, 2006. **4**(1): p. 9-12.
61. Heneghan, H., N. Miller, and M. Kerin, *Role of microRNAs in obesity and the metabolic syndrome.* Obesity reviews, 2010. **11**(5): p. 354-361.
62. Xie, H., B. Lim, and H.F. Lodish, *MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity.* Diabetes, 2009. **58**(5): p. 1050-1057.
63. Kajimoto, K., H. Naraba, and N. Iwai, *MicroRNA and 3T3-L1 pre-adipocyte differentiation.* Rna, 2006. **12**(9): p. 1626-1632.
64. Herrera, B., et al., *Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes.* Diabetologia, 2010. **53**(6): p. 1099-1109.
65. Kong, L., et al., *Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study.* Acta diabetologica, 2011. **48**(1): p. 61-69.
66. Zhao, E., et al., *Obesity and genetics regulate microRNAs in islets, liver, and adipose of diabetic mice.* Mammalian Genome, 2009. **20**(8): p. 476-485.
67. Baroukh, N., et al., *MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic β-cell lines.* Journal of Biological Chemistry, 2007. **282**(27): p. 19575-19588.
68. Tang, X., et al., *Identification of glucose-regulated miRNAs from pancreatic β cells reveals a role for miR-30d in insulin transcription.* Rna, 2009. **15**(2): p. 287-293.
69. Poy, M.N., et al., *miR-375 maintains normal pancreatic α-and β-cell mass.* Proceedings of the National Academy of Sciences, 2009. **106**(14): p. 5813-5818.
70. Zhu, H., et al., *The Lin28/let-7 axis regulates glucose metabolism.* Cell, 2011. **147**(1): p. 81-94.
71. Bak, M., et al., *MicroRNA expression in the adult mouse central nervous system.* Rna, 2008. **14**(3): p. 432-444.

72. Amar, L., et al., *MicroRNA expression profiling of hypothalamic arcuate and paraventricular nuclei from single rats using Illumina sequencing technology.* Journal of neuroscience methods, 2012. **209**(1): p. 134-143.
73. Sangiao-Alvarellos, S., et al., *Perturbation of hypothalamic microRNA expression patterns in male rats after metabolic distress: impact of obesity and conditions of negative energy balance.* Endocrinology, 2014. **155**(5): p. 1838-1850.
74. Mercader, J.M., et al., *Aberrant brain microRNA target and miRISC gene expression in the anx/anx anorexia mouse model.* Gene, 2012. **497**(2): p. 181-190.
75. Benoit, C., et al., *Early leptin blockade predisposes fat-fed rats to overweight and modifies hypothalamic microRNAs.* Journal of Endocrinology, 2013. **218**(1): p. 35-47.
76. Crépin, D., et al., *The over-expression of miR-200a in the hypothalamus of ob/ob mice is linked to leptin and insulin signaling impairment.* Molecular and cellular endocrinology, 2014. **384**(1): p. 1-11.
77. Schneeberger, M., et al., *Deletion of miRNA processing enzyme Dicer in POMC-expressing cells leads to pituitary dysfunction, neurodegeneration and development of obesity.* Molecular metabolism, 2013. **2**(2): p. 74-85.
78. Cansell, C. and S. Luquet, *Hypothalamic regulation of energy balance: a key role for DICER miRNA processing in arcuate POMC neurons.* Molecular metabolism, 2013. **2**(2): p. 55-57.
79. Vinnikov, I.A., et al., *Hypothalamic miR-103 protects from hyperphagic obesity in mice.* The Journal of Neuroscience, 2014. **34**(32): p. 10659-10674.
80. Meza-Sosa, K.F., G. Pedraza-Alva, and L. Pérez-Martínez, *microRNAs: key triggers of neuronal cell fate.* Frontiers in cellular neuroscience, 2014. **8**.
81. Kawase-Koga, Y., et al., *RNAase-III enzyme Dicer maintains signaling pathways for differentiation and survival in mouse cortical neural stem cells.* Journal of cell science, 2010. **123**(4): p. 586-594.
82. Kawahara, H., T. Imai, and H. Okano, *MicroRNAs in neural stem cells and neurogenesis.* Frontiers in neuroscience, 2012. **6**.
83. Thornton, J.E. and R.I. Gregory, *How does Lin28 let-7 control development and disease?* Trends in cell biology, 2012. **22**(9): p. 474-482.
84. Shyh-Chang, N. and G.Q. Daley, *Lin28: primal regulator of growth and metabolism in stem cells.* Cell Stem Cell, 2013. **12**(4): p. 395-406.
85. Piskounova, E., et al., *Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28.* Journal of Biological Chemistry, 2008. **283**(31): p. 21310-21314.
86. Mayr, F. and U. Heinemann, *Mechanisms of Lin28-mediated miRNA and mRNA regulation—a structural and functional perspective.* International journal of molecular sciences, 2013. **14**(8): p. 16532-16553.
87. Cimadamore, F., et al., *SOX2–LIN28/let-7 pathway regulates proliferation and neurogenesis in neural precursors.* Proceedings of the National Academy of Sciences, 2013. **110**(32): p. E3017-E3026.
88. Sangiao-Alvarellos, S., et al., *Changes in hypothalamic expression of the Lin28/let-7 system and related microRNAs during postnatal maturation and after experimental manipulations of puberty.* Endocrinology, 2013. **154**(2): p. 942-955.
89. Kawahara, H., et al., *Musashi1 cooperates in abnormal cell lineage protein 28 (Lin28)-mediated let-7 family microRNA biogenesis in early neural differentiation.* Journal of Biological Chemistry, 2011. **286**(18): p. 16121-16130.
90. Sun, T., et al., *MicroRNA let-7 regulates 3T3-L1 adipogenesis.* Molecular Endocrinology, 2009. **23**(6): p. 925-931.
91. Frost, R.J. and E.N. Olson, *Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs.* Proceedings of the National Academy of Sciences, 2011. **108**(52): p. 21075-21080.
92. Huang, Y.-W.A., et al., *Dual regulation of miRNA biogenesis generates target specificity in neurotrophin-induced protein synthesis.* Cell, 2012. **148**(5): p. 933-946.

93. Iliopoulos, D., H.A. Hirsch, and K. Struhl, *An epigenetic switch involving NF-κB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation.* Cell, 2009. **139**(4): p. 693-706.
94. Sousa-Ferreira, L., et al., *Moderate long-term modulation of neuropeptide Y in hypothalamic arcuate nucleus induces energy balance alterations in adult rats.* PLoS One, 2011. **6**(7): p. e22333-e22333.
95. De Almeida, L.P., et al., *Lentiviral-mediated delivery of mutant huntingtin in the striatum of rats induces a selective neuropathology modulated by polyglutamine repeat size, huntingtin expression levels, and protein length.* The Journal of neuroscience, 2002. **22**(9): p. 3473-3483.
96. Sousa-Ferreira, L., et al., *Proliferative hypothalamic neurospheres express NPY, AGRP, POMC, CART and Orexin-A and differentiate to functional neurons.* PloS one, 2011. **6**(5).
97. Lee, S.-T., et al., *Altered microRNA regulation in Huntington's disease models.* Experimental neurology, 2011. **227**(1): p. 172-179.
98. Zhao, C., et al., *MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling.* Proceedings of the National Academy of Sciences, 2010. **107**(5): p. 1876-1881.
99. Chow, J.C., et al., *Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction.* Journal of Biological Chemistry, 1999. **274**(16): p. 10689-10692.
100. Hayden, M.S. and S. Ghosh, *Shared principles in NF-κB signaling.* Cell, 2008. **132**(3): p. 344-362.
101. Buettner, R., J. Schölmerich, and L.C. Bollheimer, *High-fat diets: Modeling the metabolic disorders of human obesity in rodents.* Obesity, 2007. **15**(4): p. 798-808.